# PREDOMINANT PROTEINS IN HUMAN SEMEN
## AND THEIR PROTEOLYTIC PROCESSING

Hans Lilja

Malmö 1985

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Predominant proteins in human semen and their proteolytic processing

**Abstract** A protein accounting for more than 80 % of the total protein content in human seminal vesicle secretion was identified by means of an antiserum raised against a basic seminal plasma protein. The predominant protein from the seminal vesicles (HMW-SV-protein) consisted of three disulphide-linked subunits of about 52, 71, and 76 kDa, and constituted the structural matrix of coagulated semen. The HMW-SV-protein was stable in the seminal vesicle secretion, but was rapidly cleaved by prostatic proteases. Liquefaction of coagulated semen accompanied the cleavage of HMW-SV-protein to several proteins with molecular masses below 20 kDa. A series of basic cleavage products from the HMW-SV-protein constituted the characteristic group of basic proteins on agarose gel electrophoresis of liquefied semen. The predominant, distinctly basic cleavage product was isolated from liquefied semen. This 5.8 kDa basic protein, consisting of 52 amino acid residues, was devoid of cysteine, methionine, tryptophan, and leucine, but very rich in histidine.

A predominant 33 kDa glycoprotein in human prostatic secretion, known as "prostate specific antigen", was identified as an arginine-specific proteolytic enzyme of the serine protease family. The prostatic 33 kDa glycoprotein rapidly cleaved both native and carboxymethylated HMW-SV-protein, the resulting cleavage pattern being similar to that of the normal proteolysis of HMW-SV-protein in ejaculated semen. Sequence homologies with the glandular kallikreins suggest that the prostatic glycoprotein may be a kallikrein-like protease while sequence homologies with bovine high molecular weight kininogen suggest the HMW-SV-protein to be a kininogen-like substrate. The proteolysis of HMW-SV-protein was also studied in relation to a peptidase linked to prostatic organelles ("prostasomes"). It could not be settled whether this peptidase cleaved the HMW-SV-protein during liquefaction of coagulated semen, although o-phenanthroline inhibition and $Zn^{++}$ reactivation of the peptidase suggest this to be the case. Gamma-glutamyltransferase (EC 2.3.2.2) proved useful as an indicator of prostasome secretion in semen.

Key words

Adenosine triphosphatase, amino acid sequence, gamma-glutamyltransferase, inhibin, kallikrein, peptide hydrolases, protein processing, prostate, semen, seminal vesicles.

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# PREDOMINANT PROTEINS IN HUMAN SEMEN AND THEIR PROTEOLYTIC PROCESSING

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# PREDOMINANT PROTEINS IN HUMAN SEMEN
## AND THEIR PROTEOLYTIC PROCESSING

Hans Lilja

Malmö 1985

This thesis is based on the following papers:

I Lilja H, Weiber H. γ-Glutamyltransferase bound to prostatic subcellular organelles and in free form in human seminal plasma. Scand J Clin Lab Invest 1983; 43: 307-312.

II Lilja H, Weiber H. Synthetic protease inhibitors and postejaculatory degradation of human semen proteins. Scand J Clin Lab Invest 1984; 44: 433-438.

III Lilja H, Laurell C-B, Jeppsson, J-O. Characterization of the predominant basic protein in human seminal plasma, one cleavage product of the major seminal vesicle protein. Scand J Clin Lab Invest 1984; 44: 439-446.

IV Lilja H, Jeppsson J-O. Amino acid sequence of the predominant basic protein in human seminal plasma. FEBS Lett 1985; 182:181-184.

V Lilja H, Laurell C-B. Liquefaction of coagulated human semen. Scand J Clin Lab Invest 1984; 44: 447-452.

VI Lilja H, Laurell C-B. The predominant protein in human seminal coagulate. Scand J Clin Lab Invest 1985; 45: In press.

VII Lilja H. A kallikrein-like serine protease in prostatic fluid cleaves the predominant seminal vesicle protein. J Clin Invest 1985; In press.

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ABBREVIATIONS

| | |
|---|---|
| α-IB-92 | = α-Inhibin-like peptide containing 92 amino acid residues |
| ATPase | = Adenosine triphosphatase |
| DFP | = Di-isopropylfluoro-phosphate |
| EDTA | = Ethylenediaminetetraacetic acid |
| EGF | = Epidermal growth factor |
| EGTA | = (Ethylenebis(oxonitrilo))tetraacetic acid |
| GGT | = γ-Glutamyltransferase |
| HMW-SV-protein | = High molecular weight seminal vesicle protein |
| HPLC | = High pressure liquid chromatography |
| pNA | = 4-Nitroanilide |
| SDS-PAGE | = Sodium dodecyl sulphate polyacrylamide gel electrophoresis |

## INTRODUCTION

The spermatozoa mix with the secretions produced by the accessory sex glands during ejaculation. The packed spermatozoa represent less than 5 % of the human ejaculate volume (1). The secretions produced by the seminal vesicles and the prostate gland are predominant contributors to the volume of human semen. The epididymides, vasa deferentia, ampullae, the bulbo-urethral (Cowper's) glands and the urethral (Littré's) glands, all produce secretions constituting minor contributions (less than 10 %) to the ejaculate volume (2).

Both the development and secretory activity of the male accessory sex glands are strictly dependent on androgens (3). Although analogous glandular sex structures to those mentioned above occur in most mammals, certain species have additional organs, such as the coagulating gland in rodents, while structures common to most species are absent in others. The seminal vesicles are absent in the dog, for instance, in which the prostate gland is the only accessory gland. The remarkable variation in the size and secretory function of the accessory glands from one species to another is reflected in a correspondingly wide diversity in the biochemical composition of ejaculated semen (4).

The seminiferous epithelium of the testes contains the somatic, non-proliferating, Sertoli cells and the proliferating germ cells. The differentiation process leading to the formation of testicular spermatozoa, the spermatogenesis, takes more than 2 months in man (5). After release from the seminiferous epithelium, the spermatozoa mature during transport through the epididymides which takes about 12 days

(1). Testicular spermatozoa acquire fertilizing capacity during this maturation process but they remain immotile in the epididymides (1). The secretions from the accessory sex glands are ejected in a sequential manner together with the epididymal spermatozoa during the ejaculation process (6,7). The secretion from the prostate gland is emitted first, immediately followed by the sperm rich fluids from the epididymides, thereby facilitating the transport of spermatozoa, while the final portion of the human ejaculate, predominantly from the seminal vesicles, flushes the urethra free from sperm cells. The major portion of the human ejaculate either coagulates immediately after it has left the urethra (8) or is even, perhaps, emitted as a coagulate. In man, components characteristic of the blood coagulation system, such as fibrinogen or thrombin, are quite absent in the formation of the seminal coagulate (9,10). The seminal vesicle secretion is considered to contain the structural matrix of the coagulate (11). It has never been settled whether, in man, the coagulation of the vesicular secretion requires contact with other fluids, such as the prostatic fluid. However, it is probable that the mechanism is intrinsic to the secretion in itself (12). Ejaculated canine semen does not form any gel at all (4), whereas rodent semen forms a very rigid clot structure subsequent to the mixing of vesicular secretion with prostatic fluid (13). The rodent semen coagulates when basic proteins, secreted by the seminal vesicles, are polymerized via covalently formed $\gamma$-glutamyl-$\varepsilon$-lysine cross-links (14). This reaction is catalyzed by a transglutaminase (15), a protein secreted by the anterior lobe of the rodent prostate (the coagulating gland). The rodent clot structure does not readily dissolve, unlike the human

coagulate which is normally completely liquefied within 20 min after ejaculation (8). Human spermatozoa are initially trapped by the coagulate and not expressed freely, progressively motile until the clot has liquefied (8). The biochemical processes responsible for the activation of spermatozoa motility are unknown, but the motility has been reported to be distinctly enhanced by certain seminal plasma constituents such as the bovine "forward motility protein" (16), and the human membrane-coated organelles or "prostasomes" (17). Prostatic proteases are responsible for the lysis of the clot structure in man (1), but they are ill-defined. The rapid proteolytic degradation of the bulk of basic proteins continues in liquefied semen. If ejaculates are kept at room temperature for more than an hour before analysis, this auto-proteolysis of human seminal plasma proteins results in increasing concentrations of free amino acids and in severely distorted electrophoretic protein patterns.

Predominant proteins in human seminal plasma

The protein concentration of seminal plasma (i.e., the ejaculate devoid of spermatozoa) varies from 35 to 55 g/l (18). The secretions produced by the seminal vesicles and the prostate gland are the predominant contributors of proteins to human seminal plasma. Blood plasma proteins occur at low concentrations in seminal plasma, accounting for less than 5 % of its total protein content (Laurell & Lilja, unpublished). Numerous blood plasma proteins have been identified in seminal plasma and their concentrations in this fluid have been reported by several workers (see recent review by Lizana and Eneroth; (18)). Conclusions regarding the glandular origin of these

proteins have generally been based upon their relative abundance in different ejaculate fractions collected by the split technique (6,7). Albumin is present in semen at a higher concentration (0.5 to 1.4 g/l) than any other plasma protein. The prostate gland is considered to be the major site of albumin transudation (9,19).

Although, until recently, there have been few attempts to characterize the proteins in the pure secretion from the human prostate gland, the prostate gland has long been recognized for its ability to secrete acid phosphatase in high concentration (20). This glycoprotein is composed of two disulphide-linked subunits of about 50 kDa (21), and it migrates heterogeneously in the $\alpha_2$-zone during agarose gel electrophoresis at pH 8.6 (Fig. 1). Prostatic acid phosphatase has recently been shown to dephosphorylate phosphorylated tyrosine residues in proteins (22), but its physiological function in seminal plasma still remains to be established. The prostate gland also secretes enzyme-carrying, membrane-coated organelles or "prostasomes" (23), but the identity of predominant proteins other than acid phosphatase has long remained obscured. The purification and partial characterization of a 33 kDa glycoprotein, "prostate specific antigen", performed by Wang et al. (24), represents a major contribution to the identification of the predominant prostatic proteins, as does the purification and complete sequence analysis of the 11 kDa protein, "β-inhibin" (25,26) or "β-micro seminoprotein" (27), (Fig.1).

No reports have yet been published, characterizing any of the predominant proteins present in secretion collected from the glandular ducts of the seminal vesicles in man (Fig. 1). Conclusions of a

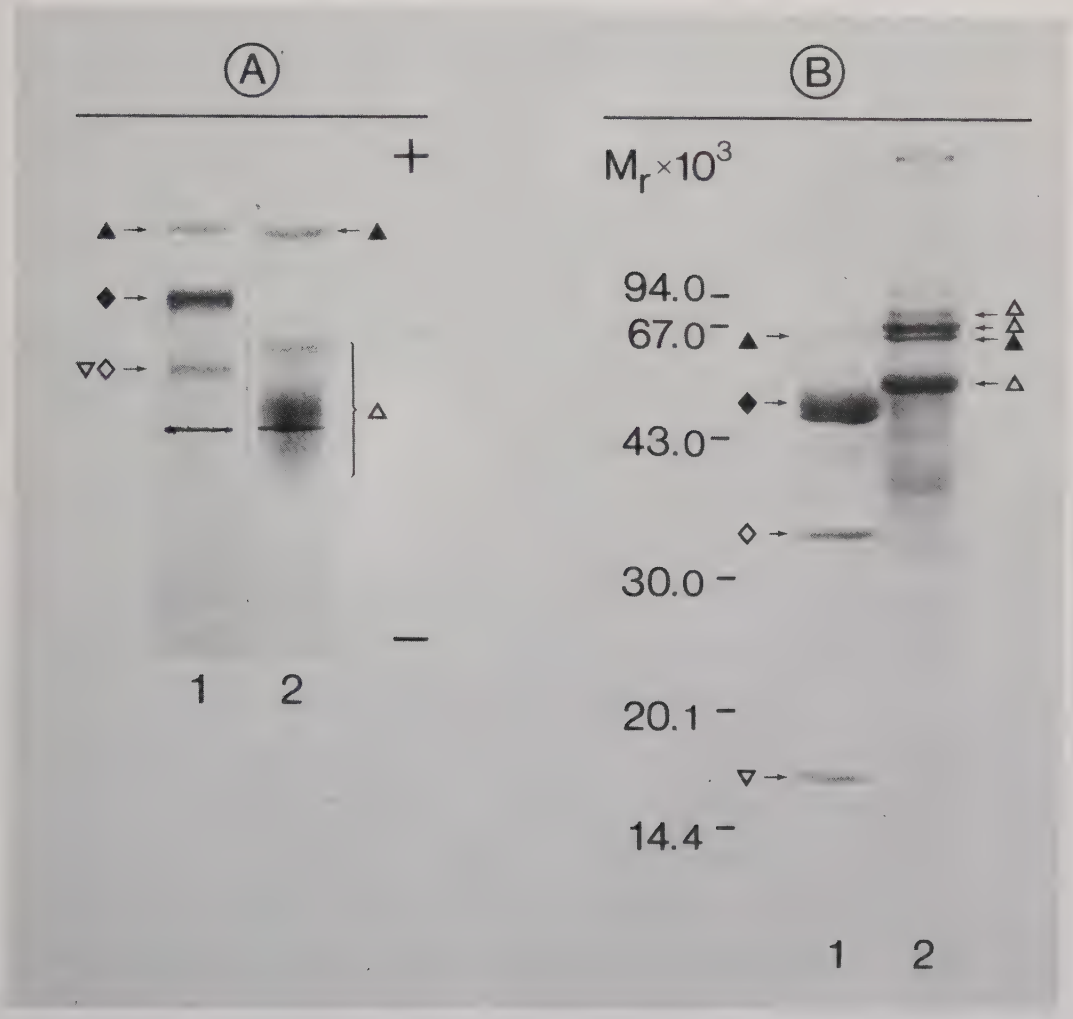


Figure 1. (A) Agarose gel electrophoresis at pH 8.6; (B) SDS-PAGE (reduced samples). The gels were stained with Coomassie R 250 after completed electrophoresis. Prostatic secretion is shown in lane 1, seminal vesicle secretion is shown in lane 2. The proteins indicated in the figure are: ▲ albumin, ◆ acid phosphatase, ◇ prostatic 33 kDa glycoprotein (prostate specific antigen), ▽ β-inhibin (β-micro seminoprotein), △ HMW-SV-protein.

vesicular origin of the group of basic proteins in liquefied semen have rested upon their abundance in the final fractions of split ejaculates (28).

## THE PRESENT INVESTIGATION

Human spermatozoa become trapped by the ejected seminal coagulate. The subsequent activation of sperm motility occurs at the time of clot lysis. The significance of this moment when sperm cells come in close contact with the predominant protein constituents of seminal plasma is not known. The highly motile sperm cells lose contact with the liquefied seminal fluid at the outset of their migration through the female genital tract. There exist no generally accepted criteria for distinguishing seminal plasma containing normally mobile spermatozoa from seminal plasma containing immobile spermatozoa. The lack of such information may in part be due to the fact that few predominant proteins secreted by the seminal vesicles and the prostate gland have been isolated, identified, and properly characterized. The purpose of the present investigation was to isolate and to characterize predominant proteins from human seminal plasma. Potential interactions between the purified proteins and spermatozoa were beyond the scope of this study.

Gamma-glutamyltransferase was evaluated as a potential marker of the prostatic organelles carrying membrane-linked peptidase, ATPase and protein kinase activities. Adequate inhibition of the high proteolytic activity in the ejaculate was a prerequisite for the characterization of proteins in seminal fluid, in particular, the

labile basic proteins originating from the seminal vesicles. Early progress in the purification from liquefied semen of the predominant, basic protein enabled antiserum against it to be produced. The identification of a vesicular high molecular mass precursor of this basic protein paved the way for further studies of the coagulation-liquefaction process subsequent to ejaculation. Studies of the purification and enzymatic characterization of a 33 kDa glycoprotein (later identified as "prostate specific antigen") were initiated to test the validity of the tentative conclusion that a serine protease, possibly related to the kallikrein family, was present in prostatic secretion and active against the previously identified high molecular mass protein from the seminal vesicles.

Gamma-glutamyltransferase linked to prostasomes in seminal plasma (I)

Membrane-coated organelles are regular constituents of seminal plasma in rabbit, ram, and man (23,29,30). In man these organelles have been termed "prostasomes", since they are secreted by the prostate gland. Prostasomes may vary considerably in size but they have an average diameter of 125 nm and a density of 1.03 g/ml (23). They carry membrane-linked $Mg^{++}$ and $Ca^{++}$ dependent ATPase (23,30), a protein kinase (31), and a peptidase which hydrolyses synthetic elastase substrates such as succinyl-$(alanine)_3$-pNA (32). A preliminary characterization at our laboratory of this proteolytic activity has shown that it was not inhibited by EDTA or by serine protease inhibitors. Although a complete inhibition was obtained with o-phenanthroline, this inhibition was reversed by $Zn^{++}$ (32).

The physiological function of prostasomes in seminal plasma has

not been established, but prostasomes devoid of the amorphous substance from the seminal vesicles may promote forward motility of washed spermatozoa (33). The possible motility promoting effect of prostasomes suggests the desirability of an indicator of prostasomes in individual samples.

Neither spermatozoa nor the epididymides are major sources of $\gamma$-glutamyltransferase (GGT; EC 2.3.2.2) in seminal plasma (34). This integral membrane protein is of prostatic origin in seminal plasma (35), and present at concentrations 200 to 500 times higher than in normal serum (36). GGT participates in the metabolism of glutathione (37) but no physiological function of the enzyme in seminal plasma has yet been established. The purpose of the study was to evaluate whether GGT could be used as a prostasome indicator suitable for routine analysis.

Ultracentrifugation (at 100,000 x g for 1 h) of pooled seminal plasma reduced the GGT-activity in the supernatant. The resulting pellet contained 13 times more GGT-activity per g protein than did the supernatant. Gradient centrifugation of the pelleted material in colloidal silica showed that the GGT-activity was linked to particles with a density of 1.03 g/ml. After gradient centrifugations of the pelleted material, peak GGT-activity coincided with the prostasome-bound ATPase and peptidase activities. Since the size range of prostasomes prevents their penetration into 1 % agarose, there was intense residual GGT-activity at the application site following agarose gel electrophoresis of the pelleted material from ultra-centrifuged seminal plasma. The glandular origin of the non-pelleted, detergent-binding GGT-activity in ultracentrifugated seminal plasma

was not established. However, in semen from vasectomized men (n = 28) there existed a very close correlation (r = 0.94) between GGT and succinyl-(alanine)$_3$-pNA hydrolysing activities, by contrast to the less close correlation between GGT and the prostatic secreted β-inhibin (r = 0.57), or to that between GGT and prostate specific antigen (r = 0.80) (Laurell & Lilja, unpublished). The postulated prostasome-linkage of seminal plasma GGT is supported by these results, and it may be concluded that the analysis of this enzyme in seminal plasma could be used to estimate the prostasome content in individual samples.

Charge shift electrophoresis showed that a minute fraction of the GGT-activity in seminal plasma was water soluble and had no detergent-binding properties. This fraction was present in pure prostatic secretion. Prostasome-linked GGT could be rendered water soluble in active form by papain digestion. A prostatic origin of the water soluble GGT fraction in seminal plasma may thus be concluded. The fraction might originate from prostasome-linked GGT and be released secondary to post-ejaculatory proteolysis. Whether the amount of this water soluble GGT fraction increases continuously in seminal plasma after the ejaculation has not been examined.

Electrophoretic mapping of the normal proteolysis of seminal plasma proteins (II, V, VI)

The electrophoretic protein patterns on agarose and SDS-polyacrylamide gels (SDS-PAGE) were analysed after the incubation of individual samples at room temperature for 0 to 24 h after the ejection of semen (Fig. 2). The protein pattern of the minute liquid portion of freshly

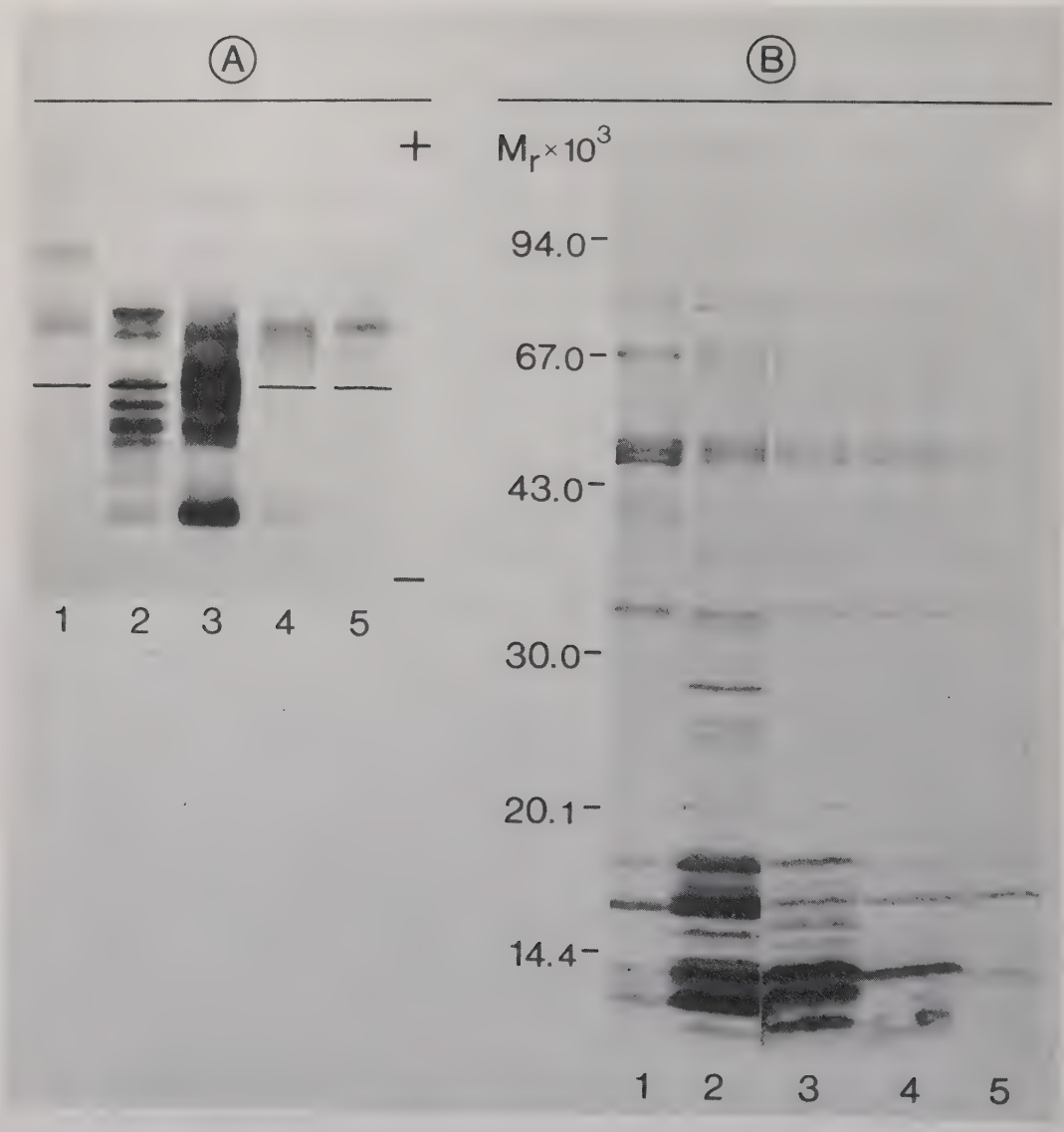


Figure 2. (A) Agarose gel electrophoresis at pH 8.6; (B) SDS-PAGE (reduced samples). The gels were stained with Coomassie R 250 after completed electrophoresis. (1) The supernatant obtained from coagulated ejaculate centrifuged at 3000 x g for 2 min. (2) Liquefied seminal plasma kept at room temperature, 30 min after ejaculation, (3) 90 min after ejaculation, (4) 6 h after ejaculation, and (5) 24 h after ejaculation.

emitted semen was very similar to that of prostatic secretion (cf Fig. 1). During liquefaction of coagulated semen, the soluble prostatic proteins were augmented by a series of basic proteins having apparent molecular masses below 20 kDa. Extended storage of liquefied seminal plasma at room temperature resulted in stepwise degradation of the basic proteins, and the protein pattern was temporarily augmented by several acidic proteins of low molecular mass. Some of these acidic proteins had a higher electrophoretic mobility than albumin on agarose. The proteins secreted by the prostate predominated in the final electrophoretic pattern of seminal plasma that had been kept at room temperature for 24 h. Edwards et al. (38) and Chantananukul & Panyim (39) have arrived at similar conclusions using two-dimensional electrophoresis and SDS-PAGE.

Effects of synthetic inhibitors on the normal proteolysis of seminal plasma proteins (II, V)

On the basis of indirect evidence, proteolytic enzymes from the human prostate have been presumed to be responsible for the lysis of coagulated semen (40). These enzymes have also been presumed to be responsible for the normal occurrence of post-liquefaction proteolysis described above. The enzymes involved are ill-defined, although some preliminary characterizations do exist (41,42). Any purification or characterization of native proteins from seminal fluids presupposes at least cursory knowledge as to which inhibitors may be used to affect the active proteases present in seminal plasma. This is particularly evident in the characterization of the group of labile basic seminal plasma proteins which are presumed to originate from the seminal

vesicles (28,32). Edwards et al. (38) reported in 1981 that a combination of phenylmethylsulfonyl fluoride, a serine protease inhibitor, and EDTA, a chelating compound, may inhibit the proteolysis of basic proteins in seminal plasma. However, their results were not conclusive with regard to which of the two agents was the effective one, or whether both were necessary (38). The patent lack of reported data on such aspects of seminal plasma proteolysis prompted our own study.

Liquefaction of coagulated semen: Coagulated semen that was acidified to about pH 5 (with acetic acid) liquefied completely at 37°C. Seminal coagulate made up to 10 mmol/l with di-isopropyl-fluoro-phosphate (DFP), 100 mmol/l with benzamidine, 30 mmol/l with $Na_2EDTA$, 50 mmol/l with $Na_2EGTA$, or 30 mmol/l with iodoacetamide also liquefied completely. A very high concentration of o-phenanthroline-HCl (50 mmol/l) inhibited liquefaction of coagulated semen, an effect that was partially reversed by the subsequent addition of $Zn^{++}$ to a final concentration of 3 to 6 mmol/l.

Proteolysis of liquefied semen: The electrophoretic protein patterns of liquefied semen samples kept at room temperature for 0 to 24 h were most effectively stabilized by the addition of the irreversible serine protease inhibitor, DFP (10 mmol/l). The addition of exogenous thiol reagents such as iodoacetamide (30 mmol/l) or the chelating compound o-phenanthroline (50 mmol/l) proved almost as effective. The degradation of the series of basic, low molecular mass proteins in seminal plasma was retarded by benzamidine at a

concentration of 100 mmol/l, although this group of basic proteins was completely degraded after incubation of semen for 24 h at room temperature. Neutrally buffered $Na_2EDTA$ or $Na_2EGTA$ were much less effective than benzamidine, although some retardation of the proteolytic degradation could be demonstrated.

The high inhibitory effect obtained by different serine protease inhibitors suggests that serine protease(s) may be mainly responsible for the rapid post-liquefaction proteolysis in human semen. The inhibitory effect achieved by thiol reagents might be explained by irreversible inactivation of serine proteases by alkylation; alternatively, were it not for the fact that cysteine proteases usually occur intracellularly (43), the effect might be accounted for by inhibition of cysteine proteases. Seminal coagulate liquefied at pH 5, a finding at variance with a previous report (8). The addition of o-phenanthroline to coagulated semen enables the native protein composition to be studied before liquefaction has occurred. Liquefaction of coagulated semen is inhibited and reactivated in a manner that is similar to the inhibition and reactivation of the prostasome-linked peptidase activity. No conclusions can be drawn as to any involvement of this peptidase in the lysis reaction, owing to the possible presence in our experiments of contaminating proteolytic activities in the pelleted material obtained after ultra-centrifugation of seminal plasma. The metal ion dependence of the prostasome-linked peptidase activity, also reported on by Laurell et al. in (32), needs to be studied further to make it comprehensible. The proposed participation of the EDTA-sensitive, collagenase-like peptidase in the liquefaction process (42) seems improbable in view of

our results.

Purification and characterization of the predominant basic protein in liquefied semen (III, IV)

The predominant, distinctly basic protein in seminal plasma was, due to its lability, purified from this fluid in the presence of serine protease inhibitors (DFP and benzamidine). Purified basic protein was obtained in a relatively high yield with a procedure consisting of three chromatographic steps, the predominant basic protein being identified by agarose gel electrophoresis after each step. An initial Heparin-Sepharose chromatography was followed by gel filtration on Biogel P 60. Polyacrylamide was a better gel matrix for the gel filtration than were either agarose or dextran, although the elution pattern was indistinct unless denaturing conditions were used. The indistinct elution profile may have been caused by protein interaction with negatively charged groups remaining in the gel matrices. The recovery after the second step was about 50 %. Reversed phase chromatography on a $C_1/C_8$ column was used for the final purification, the predominant basic protein eluting from this column as a homogeneous peak. The purity of the material in this peak was confirmed by SDS-PAGE, agarose gel electrophoresis, and by $NH_2$-terminal sequence determination which gave a single sequence.

The purified protein had a pI value above 9, and contained less than 0.2 mol hexose per mol protein according to the anthrone reaction (Lilja, unpublished), and less than 0.2 mol hexosamine per mol protein according to amino acid analysis. The molecular mass was determined by several independent methods. SDS-PAGE gave the protein an apparent

mass above that of $\beta_2$-microglobulin (11.8 kDa). A mass of about 15 kDa was obtained from HPLC gel filtration on a TSK G 2000 SW column equilibrated in 0.05 mol/l $NH_4HCO_3$, whereas gel filtration in 6 mol/l guanidine-HCl on Sephacryl S 200 gave the purified protein a mass of 6 kDa. The mass of 6 kDa is in close agreement with that calculated from the amino acid sequence of the protein (5.8 kDa). The existence of a dimeric form of the purified protein, that is remarkably stable to denaturing conditions, could explain the divergent results obtained by SDS-PAGE and gel filtrations, a conclusion supported by the bimodal elution pattern of the purified protein on HPLC gel filtration in 6 mol/l guanidine-HCl. The very high positive charge of the purified protein could provide another, but less probable, explanation for the divergent result on SDS-PAGE, as basic proteins have been reported to show a lower mobility on SDS-PAGE than might be expected from their masses (44). However, the basic protein, aprotinin (6.4 kDa), had a considerably higher mobility on SDS-PAGE than either our purified basic protein or $\beta_2$-microglobulin.

Amino acid sequence of the predominant basic protein (IV)

The amino acid sequence of the predominant basic protein was deduced from automated $NH_2$-terminal sequencing of the intact protein and purified fragments. The COOH-terminal sequence was confirmed by carboxypeptidase Y digestion. All peptides isolated were consistent with the deduced sequence of 52 amino acid residues. The protein is devoid of cysteine, methionine, leucine, and tryptophan, but it is very rich in histidine in its $NH_2$-terminal half.

The $NH_2$-terminal sequence of the predominant basic protein is

identical to the 31 $NH_2$-terminal amino acids determined in a 5 kDa seminal plasma peptide (45). This peptide has been reported to inhibit the secretion of rat pituitary follicle-stimulating hormone (45). It may represent a degradation product of the predominant 5.8 kDa basic protein. This conclusion is based on the fact that, although the basic protein is very labile in the seminal plasma, the inhibin-like peptide was purified in the absence of protease inhibitors during all procedures reported (45,46). Hao Li et al. (47) recently reported the amino acid sequences of two additional peptides with inhibin-like activity. One of these peptides was identical to our 5.8 kDa basic protein. A larger peptide ($\alpha$-IB-92), consisting of 92 amino acid residues, had also been isolated. Our 5.8 kDa basic protein constituted the COOH-terminal portion of this larger peptide.

The $NH_2$-terminal of our sequenced basic protein is to 34 % identical with one portion of the histidine-rich fragment of bovine high molecular weight kininogen (Fig. 3). This histidine-rich fragment mediates the surface binding of kininogen (51). The identity with the basic protein is 41 % in an overlap with another portion of this kininogen fragment (Fig. 3). There is 28 % identity between the 67 $NH_2$-terminal amino acid residues of the $\alpha$-IB-92 peptide (47) and residues Nos. 425 to 492 of bovine high molecular weight kininogen (48,49). The $NH_2$-terminal of our protein also provides relatively high sequence homologies with human histone 2 A (30 %) and DNA dependent RNA-polymerase (34 %). The COOH-terminal sequence of the basic seminal plasma protein provides two different overlaps with the COOH-terminal of the epidermal growth factor (EGF) precursor (52). Nine of 25 residues (36 %) are identical in the first overlap, whereas

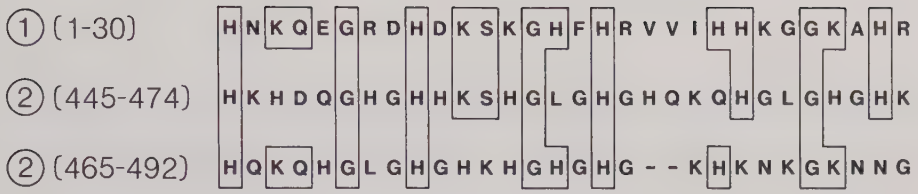


Figure 3. Comparison of sequences in (1) the $NH_2$-terminal of the 5.8 kDa basic protein, and (2) in the histidine-rich fragment of bovine high molecular weight kininogen. The sequence reported by Han et al (48,49) has been used, the numbering is according to Kitamura et al. (50). The sequences are presented in the standard IUPAC, one-letter code for amino acid residues.

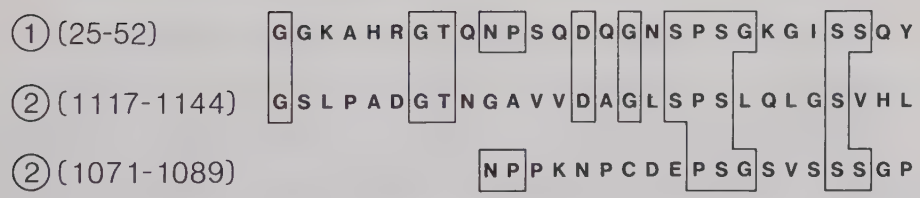


Figure 4. Comparison of the sequences in (1) the COOH-terminal of the 5.8 kDa basic protein, and (2) in the EGF-precursor (52). The sequences are presented in the standard IUPAC, one-letter code for amino acid residues.

7 of 17 residues (41 %) are identical in a second overlap (Fig. 4). This portion of the EGF-precursor has been proposed to be a cytoplasmic domain (53,54) and it is situated between the COOH-terminal and the growth factor.

Identification of a predominant protein in seminal vesicle secretion as the high molecular mass precursor of the 5.8 kDa basic protein (III, VI)

A polyclonal antiserum was raised against the purified predominant basic protein. This antiserum recognized the purified basic protein, basic proteins in seminal plasma, and a high molecular mass protein in seminal vesicle secretion, but did not recognize proteins in other body fluids. The positive reactions were eliminated when the antiserum was adsorbed with the purified basic protein.

One predominant protein, which accounted for more than 80 % of the total protein present in seminal vesicle secretion after reduction, consisted of three subunits on SDS-PAGE that were all recognized by our antiserum. Of these subunits, one had an apparent mass of 52 kDa, another of 71 kDa, and the third of 76 kDa. Unreduced seminal vesicle secretion contained disulphide-linked high molecular mass complexes of this protein (HMW-SV-protein). The 52, 71, and 76 kDa bands were not labile in the seminal vesicle secretion, but were rapidly cleaved by the action of prostatic proteases. This cleavage liberated several basic low molecular mass proteins, which were indistinguishable from the basic proteins in liquefied seminal plasma. The 5.8 kDa predominant basic protein was one of the low molecular mass proteins obtained during this cleavage. In addition, there

appeared acidic cleavage products that, unlike the basic low molecular mass proteins, did not react with our antiserum.

Identification of the HMW-SV-protein as the predominant protein in seminal coagulate (V, VI)

Seminal coagulate, made up to 50 mmol/l with o-phenanthroline-HCl and washed free of soluble components, was dissolved by 2 to 3 mol/l of guanidine-HCl. Unreduced coagulate contained high molecular mass complexes of the HMW-SV-protein. Dithiothreitol added to seminal coagulate did not liquefy the clot, although the disulphide-linked complexes between HMW-SV-proteins disintegrated. Reduced coagulate was subsequently shown to contain the three (52, 71, and 76 kDa) bands of the HMW-SV-protein as its predominant constituents. The coagulate also contained cleavage products of the HMW-SV-protein that had masses from 20 to 50 kDa. The intact HMW-SV-protein was absent in the minor liquid portion of freshly emitted ejaculate. Liquefaction of coagulated semen occurred simultaneously with cleavage of the intact HMW-SV-protein to low molecular mass proteins. These results suggest that the HMW-SV-protein constitutes the structural protein of the clot. The non-covalent linkages between HMW-SV-proteins were essential to the clot structure, and thus transglutaminase-mediated covalent cross-linking may not be involved in the formation of a human seminal coagulate. This suggestion receives support from the absence of factor XIII in human seminal plasma previously reported (10), and from our confirmatory finding that transglutaminase-activity was not detectable in human seminal fluids.

Chantananukul & Panyim (39) and Chaistitvanich & Boonsaeng (55)

have reported on the identification of predominant protein bands of about 50 and 70 kDa in reduced seminal coagulate but the glandular origin of these proteins was not established. Both studies reported the conversion of these 50 and 70 kDa bands to low molecular mass proteins during liquefaction of the coagulate. Chaistitvanich & Boonsaeng (55) also reported that the 50 and 70 kDa bands formed disulphide-linked complexes, although their conclusion was that the disulphide bonds were essential for the clot structure.

The basic clot-forming proteins from the seminal vesicles are devoid of cysteine in the rat (56,57) and in the guinea pig (58), and contain very little tryptophan and methionine, but high percentages of glutamic acid and lysine. Essentially, these properties are shared by the $\alpha$-IB-92 peptide (47), and the predominant basic 5.8 kDa cleavage product of the HMW-SV-protein. The HMW-SV-protein may contain cysteine as indicated by its susceptibility to reducing agents. The presence of cysteine in the seminal clot provides additional support for this assumption. No further comparison of the clot-forming proteins is possible at present, owing to the lack of sequence data on the vesicular proteins.

Identification of the prostatic serine protease that cleaves the HMW-SV-protein (VII)

Canine prostatic secretion contains a predominant glycoprotein which accounts for more than 90 % of the total protein in this fluid (59). The synthesis of this 29 kDa protein is strictly dependent on androgens (59). The protein is an arginine-cleaving serine protease with a pH optimum between 8.0 and 8.5 (60,61). Although no

physiological substrate has been identified for this enzyme, results from partial amino acid sequencing indicate that the enzyme is more closely related to glandular kallikreins than to trypsin or chymotrypsin (62).

Our previous results (II, VI) indicated that a serine protease might be present in human seminal plasma and active against the HMW-SV-protein. Proteases active against the HMW-SV-protein were also found to be present in prostatic secretion (VI). Similarities to the canine protease with regard to molecular masses suggested that a 33 kDa protein, present at high concentration in human prostatic secretion (Fig. 1), might be a species analogue to the canine protease.

The 33 kDa prostatic protein was purified using a system consisting of four chromatographic steps, the overall yield from which was about 20 %, the purified protein being more than 90 % free from contaminants as judged by SDS-PAGE. Active site titration with 4-nitrophenylguanidobenzoate showed that 66 % of the purified protein had enzymatic activity. Sequence analysis of the purified prostatic protein showed that the 15 $NH_2$-terminal residues were identical with the reported $NH_2$-terminal of a 33 kDa glycoprotein called "prostate specific antigen" (Fig. 5). The $NH_2$-terminal sequence of this protein (63), consisting of 68 amino acid residues, contains a portion around His 41 that is identical with one part of the catalytic triad of serine proteases (64). Our purified protein incorporated ($^3H$)-DFP, and DFP destroyed its functional activity. The substrate specificity was restricted to arginine- and lysine-containing substrates but higher for the former. All synthetic substrates tested

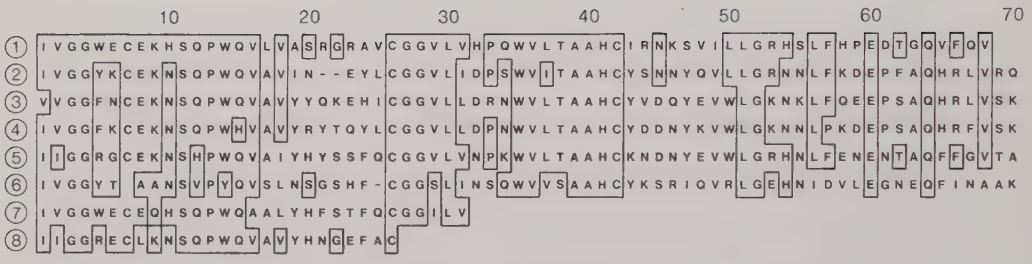


Figure 5. Comparison of the $NH_2$-terminal sequence of (1) prostate specific antigen (i.e. the purified prostatic 33 kDa glycoprotein) with the $NH_2$-terminal sequences of (2) rat tonin (66); (3) epidermal growth factor-binding protein (67); (4) the $\gamma$-subunit of nerve growth factor (68); (5) porcine pancreatic kallikrein (69); (6) porcine trypsin (70); (7) human urinary kallikrein (71); and (8) canine prostatic arginine esterase (62). The sequences are presented in the standard IUPAC, one-letter code for amino acid residues.

were poor substrates for the purified protein. The reported rates of hydrolysis of proline-phenylalanine-arginine-pNA and benzoyl-arginine-pNA by the canine protease (65) are very similar to the $V_{max}$ rates determined for the purified protein. The sequence of the purified protein is to 58 % identical with the reported 26 $NH_2$-terminal residues (62) of the canine protease (Fig. 5). These results strongly suggest that the human 33 kDa prostatic glycoprotein, identified as prostate specific antigen, is a human species analogue to the canine prostatic serine protease.

Both native and carboxymethylated HMW-SV-protein was rapidly cleaved by the purified prostatic protein, during which the 5.8 kDa basic protein was slowly released. The cleavage pattern of the intact HMW-SV-protein was very similar to the normal proteolytic cleavage pattern of the HMW-SV-protein in seminal plasma. The HMW-SV-protein might be concluded to be a (the) physiological substrate for the human prostatic protease (prostate specific antigen). The physiological substrate from the seminal vesicles has limited sequence homologies with high molecular weight kininogen and the EGF-precursor.

The $NH_2$-terminal sequence of prostate specific antigen (63), i.e., the purified prostatic protein, is very similar to the $NH_2$-terminal sequences of several serine proteases (Fig. 5). The protein may be classified as an enzyme of the serine protease family. The specificity of our purified protein, much greater for arginine- than for lysine-containing substrates, and the sequence homologies indicate that the prostatic glycoprotein is not identical with the glandular kallikreins but is more closely related to this group of serine proteases than it is to trypsin.

Identification of the predominant low molecular mass protein in prostatic secretion

Beta-inhibin (25,26), or β-micro seminoprotein (27), is an 11 kDa prostatic protein (27,72). In immunocytochemical studies Tsuda et al. found that this protein occurs on the sperm surface (73). This protein has not yet been accorded any other function in seminal plasma, but it may inhibit the secretion of rat pituitary follicle-stimulating hormone (26). We have identified this protein in the electrophoretic protein pattern of prostatic fluid (Fig. 1) by $NH_2$-terminal sequence analysis of the purified protein (Lilja, unpublished). It may be concluded that this protein is one of the three predominant proteins secreted by the human prostate gland (Fig. 1).

## GENERAL DISCUSSION

The predominant protein (HMW-SV-protein), which accounted for more than 80 % of the total protein present in seminal vesicle secretion, was identified as structural matrix of coagulated semen. Chantananukul & Panyim (39) reported that such a predominant protein in freshly ejected semen was phosphorylated if the ATPase activity had been inhibited. They could also show that the phosphorylated proteins in liquefied semen, analysed 30 min after ejaculation, consisted of low molecular mass proteins. The electrophoretic data presented by Chantananukul & Panyim (39) agree with the interpretation that the phosphorylated products are identical with our HMW-SV-protein and the low molecular mass proteins liberated from HMW-SV-protein during clot lysis. Differences in the degree of phosphorylation of the low

molecular mass proteins derived from the HMW-SV-protein may be partly responsible for the striking charge heterogeneity demonstrated for these cleavage products. The probable phosphorylation of HMW-SV-protein raises additional questions regarding possible interactions between this protein and the prostasomes. Such an interaction could not be conclusively demonstrated in connection with potential substrates for the prostasome-linked peptidase activity. The prostasome-linked ATPase and protein kinase activities are, however, very strong candidates for involvement in the reported phosphorylation-dephosphorylation reactions. Serine and threonine residues served as an accepting amino acid for the prostasome-bound protein kinase, and histone was an excellent exogenous phosphoryl group acceptor in these experiments (31). The sequence homology between the 5.8 kDa basic protein and human histone 2A thereby provides additional support to the conclusion that HMW-SV-protein or its cleavage products may serve as phosphoryl group acceptors. The accepting amino acids on the HMW-SV-protein are unknown. Protein phosphorylation-dephosphorylation reactions may be important mechanisms for regulation of cellular functions (74). Prostatic acid phosphatase, which was recently reported to dephosphorylate phosphorylated tyrosine residues in proteins (22), may be another candidate for involvement in these reactions.

The 5.8 kDa distinctly basic protein in seminal plasma was a predominant cleavage product liberated from the HMW-SV-protein during clot lysis. The antiserum raised against the 5.8 kDa basic protein recognized a large number of cleavage products from the HMW-SV-protein. The native HMW-SV-protein was cleaved stepwise but the

transformation of one band into another could not be followed due to the complexity of the cleavage pattern. The large number of fragments recognized by our antiserum might suggest that, like the EGF-precursor (52), the HMW-SV-protein may contain several repetitions or partial repetitions of the 5.8 kDa basic protein sequence. The sequence of 40 amino acid residues reported to precede the $NH_2$-terminal of our 5.8 kDa basic protein (47) contains three short portions which are repeated in the 5.8 kDa basic protein sequence. Two of these repetitions, each consisting of four residues, are hydrophilic and occur at corresponding positions relative to the $NH_2$-terminals in the sequences of the $\alpha$-IB-92 peptide and the 5.8 kDa basic protein.

High molecular weight kininogen forms complexes on negatively charged surfaces with the Hageman factor and prekallikrein (75). The histidine-rich fragment of kininogen mediates the surface binding of this complex during the intrinsic activation of blood coagulation (51). The sperm cells come in close contact with the clot-forming HMW-SV-protein during ejaculation. The sequence homology with bovine kininogen suggests that the most basic cleavage product of the HMW-SV-protein might mediate surface binding to the spermatozoa. The occurrence and possible effects of such interactions are now being studied.

Various proteolytic enzymes from human seminal plasma have been partially characterized, including two plasminogen activators (76), one pepsin-like enzyme (77), a collagenase-like peptidase (42), and a neutral protease called seminin (41). These enzymes have been thought to originate from the prostate gland but their physiological

substrates have not been identified. Both seminin and the collagenase-like peptidase have been suggested to participate in the clot lysis of human semen (12,41,42). It is not possible, from available data on these enzymes, to establish whether any of them might be identical with our prostatic 33 kDa glycoprotein. Tissue kallikrein has been demonstrated at very low concentration (10 μg/l) in human seminal plasma (78). Enzymatically active tissue kallikrein has been purified from human seminal plasma by using kallikrein-specific IgG Sepharose (79). The purified kallikrein had properties that were strikingly similar to those of human urinary kallikrein (80,81).

The prostatic 33 kDa glycoprotein, also known as prostate specific antigen (63), may participate in the clot lysis of human semen although serine protease inhibitors such as DFP failed to inhibit liquefaction of freshly ejected semen. This assumption is supported by a preliminary observation that inactivation of the enzyme by DFP may be incomplete even after 10 to 15 min at 200 times molar excess of the inhibitor (Lilja, unpublished). The participation of the prostasome-linked peptidase in the HMW-SV-protein proteolysis and in the parallel lysis reaction was not conclusively demonstrated but may be properly studied if the prostasome-containing pellets are inactivated with DFP.

The prostatic 33 kDa glycoprotein is a serine protease that slowly releases the 5.8 kDa basic protein during cleavage of the HMW-SV-protein. It may be suggested, from the reported sequence of the α-IB-92 peptide (47), that the prostatic protease cleaves COOH-terminal of the tripeptide sequence Gln-Leu-Leu. This cleavage may liberate our 5.8 kDa basic protein. The human prostatic protease was

more closely related to glandular kallikreins than to trypsin. Two of the proteases in the glandular kallikrein family, the γ-subunit of nerve growth factor and the epidermal growth factor binding protein, are involved in the processing of growth factors (82,83). The possibility can not be excluded of growth factor-related effects mediated by fragments liberated from the HMW-SV-protein during cleavage by the prostatic glycoprotein.

## GENERAL SUMMARY

A protein accounting for more than 80 % of the total protein content in human seminal vesicle secretion was identified by means of an antiserum raised against a basic seminal plasma protein. The predominant protein from the seminal vesicles (HMW-SV-protein) consisted of three disulphide-linked subunits of about 52, 71, and 76 kDa, and constituted the structural matrix of coagulated semen. The HMW-SV-protein was stable in the seminal vesicle secretion, but was rapidly cleaved by prostatic proteases. Liquefaction of coagulated semen accompanied the cleavage of HMW-SV-protein to several proteins with molecular masses below 20 kDa. A series of basic cleavage products from the HMW-SV-protein constituted the characteristic group of basic proteins on agarose gel electrophoresis of liquefied semen. The predominant, distinctly basic cleavage product was isolated from liquefied semen. This 5.8 kDa basic protein, consisting of 52 amino acid residues, was devoid of cysteine, methionine, tryptophan, and leucine, but very rich in histidine. This basic protein is identical to a recently reported peptide called α-inhibin-52 which may inhibit

the secretion of rat pituitary follicle-stimulating hormone.

A predominant 33 kDa glycoprotein in human prostatic secretion was isolated, and identified as an arginine-specific proteolytic enzyme of the serine protease family. $NH_2$-terminal sequence analysis showed that it was identical to a protein known as "prostate specific antigen". The prostatic 33 kDa glycoprotein rapidly cleaved both native and carboxymethylated HMW-SV-protein, the resulting cleavage pattern being similar to that of the normal proteolysis of HMW-SV-protein in ejaculated semen. Sequence homologies with the glandular kallikreins suggest that the prostatic glycoprotein may be a kallikrein-like protease while sequence homologies with bovine high molecular weight kininogen suggest the HMW-SV-protein to be a kininogen-like substrate. The proteolysis of HMW-SV-protein was also studied in relation to a peptidase linked to prostatic secretory organelles known as "prostasomes". It could not be settled whether this peptidase cleaved the HMW-SV-protein during liquefaction of coagulated semen, although o-phenanthroline inhibition and $Zn^{++}$ reactivation of the peptidase might suggest this to be the case. Gamma-glutamyltransferase (EC 2.3.2.2) proved useful as an indicator of prostasome secretion in seminal plasma.

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# γ-Glutamyltransferase bound to prostatic subcellular organelles and in free form in human seminal plasma

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Lilja, H. & Weiber, H. γ-Glutamyltransferase bound to prostatic subcellular organelles and in free form in human seminal plasma. *Scand. J. clin. Lab. Invest.* **43,** 307–312, 1983.

Most of the γ-glutamyltransferase occurring in semen was found to be bound to the membranes of prostatic organelles, as is the case with $Mg^{++}$–$Ca^{++}$-dependent ATPase, and $Zn^{++}$-dependent peptidase hydrolysing succinyl $(alanine)_3$-paranitroanilide. Organelle-bound γ-glutamyltransferase was released in a water-soluble form by papain. Charge shift electrophoresis revealed the presence in seminal fluid of a small additional amount of γ-glutamyltransferase that is unbound and water-soluble, but also of prostatic origin. After papain treatment there was no sign of the organelle-bound $Zn^{++}$-dependent peptidase activity, nor of the $Mg^{++}$–$Ca^{++}$ dependent ATPase activity, both having been inactivated by the papain. Both organelle-bound γ-glutamyltransferase and $Zn^{++}$-dependent peptidase could be rendered soluble in active form with the non-ionic detergent Triton X-100.

*Key-words:* adenosine triphosphatase, γ-glutamyl transpeptidase, membrane-bound proteins, peptidohydrolase, prostate, semen.

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γ-Glutamyltransferase (EC 2.3.2.2), GGT, is an integral membrane enzyme that catalyzes the transfer of γ-glutamyl groups of glutathione, and other γ-glutamyl peptides, to dipeptides and amino acids. GGT is active in the γ-glutamyl cycle and is generally assumed to play an important part in the cellular uptake of amino acids [13]. The enzyme has been detected in most parts of the male genital tract [1, 4, 5]. Concentrations of GGT in human seminal plasma are 200–500 times higher than those in normal serum [6, 16]. GGT is present in particulate form in human ejaculate [10] but neither spermatozoa nor the epididymis are major sources of GGT [17, 18]. Analysis of GGT activities on split ejaculates, and of seminal plasma from a subject with aplasia of the deferent ducts and seminal vesicles, have shown the prostate to be the major source of GGT in human seminal plasma [21].

Subcellular organelles of prostatic origin are normally present in human seminal plasma. These organelles contain a $Mg^{++}$–$Ca^{++}$-dependent ATPase and a $Zn^{++}$-dependent peptidase [3, 11, 15]. This study deals with the distribution of GGT between subcellular prostatic organelles and fluid phase in human seminal plasma, using $Zn^{++}$-dependent peptidase-hydrolysing succinyl $(alanine)_3$-paranitroanilide, and $Mg^{++}$–$Ca^{++}$-dependent ATPase, as reference enzymes.

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## MATERIAL AND METHODS

*Chemicals.* Succinyl (alanine)$_3$-paranitroanilide (Suc(Ala)$_3$pNA) was purchased from Protein Research Foundation (Minoh, Shi, Osaka, Japan); Percoll® from Pharmacia (Uppsala, Sweden); Adenosin-5-triphosphate (ATP) and L-γ-glutamyl-paranitroanilide from Boehringer Mannheim GmbH (West Germany); Triton X-100, papain type IV and L-cysteine from Sigma Chemicals (St Louis, MO, USA); Sodium deoxycholate from E. Merck (Darmstadt, West Germany); Cetyltrimetylammoniumbromide (CTAB) from Schuchardt GmbH & Co (Munich, West Germany); N-l-naphtyletylenediamine dihydrochloride and N-glycylglycine from the British Drug Houses Ltd, Chemicals (Poole, England). All other chemicals were of reagent grade.

*Samples.* Human semen was acquired from the fertility laboratories in Malmö, Sweden. Seminal plasma was obtained after centrifugation of semen at 800 × *g* for 15 min, the supernatant being either used immediately or frozen at −18°C within 2–4 h of ejaculation.

Semen from one subject with pure prostatic secretion (azoospermia, no fructose content, low volume, and aplasia of the seminal vesicles as verified by computer tomography) was used in ultracentrifugation and electrophoretic studies.

*Protein determination.* Total protein content was determined according to Lowry *et al.* [12].

*Agarose gel electrophoresis.* This was run in 0.075 mmol/l barbital buffer, pH 8.6, with 2 mmol/l calcium lactate in agarose gels, 10 g/l [8].

*Charge shift electrophoresis.* Charge shift electrophoresis was performed with the addition of sodium deoxycholate + Triton X-100, CTAB + Triton X-100, or of Triton X-100 alone, to samples and agarose gels. The buffer was 0.075 mmol/l barbiturate, pH 8.6, containing 2 mmol/l calcium lactate and sodium deoxycholate or CTAB [7]. The gels were stained for GGT activity after charge shift electrophoresis.

*GGT staining.* The staining method described by Kok *et al.* [9] was modified and improved in accordance with [2]. A 1% (w/v) agarose gel (150 mmol/l Tris-HCl buffer pH 8.3 containing 4 mmol/l L-γ-glutamylparanitroanilide, 75 mmol/l glycylglycine, 10 mmol/l $MgCl_2$, 15 mmol/l naphtyletylenediamine and 14 mmol/l $NaNO_2$) was moulded shortly before the electrophoresis was finished. The substrate gel was applied on top of the electrophoresis gel after completed electrophoresis, the combined gels then being incubated for 20 min at 37°C. The substrate gel was then removed, and the electrophoresis gel soaked in 12.5% (w/v) trichloroacetic acid for 30–60 sec, after which the GGT activity was visible as purple coloured bands.

*GGT activity* was determined as recommended by the Committee on Enzymes of the Scandinavian Society for Clinical Chemistry [2].

*ATPase activity* was determined essentially as described by Ronquist *et al.* [15] but with a slight modification [11].

*$Zn^{++}$-dependent Suc (Ala)$_3$pNA hydrolysis* was determined as described in [11].

*Ultracentrifugation.* Test tubes (capacity 10 ml), each containing 6 ml pooled seminal plasma, were centrifuged at 100,000 × $g_{av}$ at 4°C for 2 h. The supernatants were either separated from the pellets or collected as twelve 0.50 ml fractions, starting from the top. The pellets obtained by ultracentrifugation were re-suspended in 0.50 ml 0.05 mol/l Tris-HCl buffer, pH 7.4, containing 0.15 mol/l NaCl.

*Gradient centrifugation.* One 0.20 ml portion of the pellet suspension was treated with papain as outlined below, and another was treated with 1% (v/v) Triton X-100 for 1 h at room temperature. Parallel to a third 0.20 ml untreated pellet suspension, they were layered onto 20–50% (w/v) sucrose gradients and centrifuged at 100,000 × $g_{av}$ for 12 h at 4°C. One ml fractions were collected by pumping 60% (w/v) sucrose through a perforation in the bottom of the tubes and draining the tops through a stopper with a conical inlet.

A pellet suspension of 0.20 ml was layered onto a 10 ml self-generating silica gradient [14] in 0.15 mol/l NaCl with a starting density of 1.09 g/ml. The gradient was centrifuged at 30,000 × $g_{av}$ for 3 h at 4°C. One ml fractions were collected as above.

*Papain treatment.* A volume of 0.20 ml pellet suspension, with a protein content of 1.8 mg, was treated with 0.4 mg papain, 20 μl 20 mmol/l $Na_2$ EDTA, and 20 μl 50 mmol/l freshly prepared cysteine solution for 3 h at 37°C.

### *Density determination*

The density determinations of both Percoll® and sucrose concentrations were done with a Universal-Sugar-Refractometer from Schmidt-Haensch, Berlin, West Germany.

## RESULTS

*Ultracentrifugation.* Centrifugation of 6 ml pooled seminal plasma, at 100,000 × *g* at 4°C for 2 h, reduced GGT activity in the supernatant, by between 36% and 60% ($n=4$) of that in the original seminal plasma. GGT activities and protein concentrations of a fractionated supernatant are given in Table I. GGT activity per g protein was considerably higher in the pellet suspension than in seminal plasma or in supernatant fractions (Table 1). The major part of both the $Suc(Ala)_3pNA$ hydrolytic and the $Mg^{++}$–$Ca^{++}$-dependent ATPase activities were recovered in the pellet.

Pooled seminal plasma, treated with 5% Triton X-100 before being centrifuged at 100,000 × *g* for 2 h, retained all GGT and $Suc(Ala)_3pNA$ hydrolytic activities in the supernatant. No $Mg^{++}$–$Ca^{++}$-dependent ATPase activity was recovered in this supernatant.

*Gradient centrifugation.* Peak enzyme activities of GGT and $Suc(Ala)_3pNA$ hydrolysis in the pellet suspension were found at a density of 1.03 g/ml after centrifugation in colloidal silica gradients.

After sucrose gradient centrifugation of the pellet suspension fraction 6, with a sucrose concentration of 40%, was found to contain practically all $Suc(Ala)_3pNA$ hydrolysing activity, and the peak GGT and $Mg^{++}$–$Ca^{++}$-dependent ATPase activities (Fig. 1a). Sucrose gradients were used instead of silica gradients in subsequent experiments because of the distinct separation of the organelle fraction from the contaminating seminal plasma proteins (Fig. 1).

When non-ionic detergent treated pellet suspension (1% Triton X-100) was centrifuged on a 20–50% sucrose gradient both GGT and $Zn^{++}$-dependent peptidase activities were found on top of the gradient, and the $Mg^{++}$–$Ca^{++}$-dependent ATPase activity had been inactivated (Fig. 1b). In a similar experiment after papain treatment of the pellet suspension the GGT activity was found in all fractions from the top down to the fraction displaying peak activity in Fig. 2a. The $Suc(Ala)_3pNA$-hydrolysing and $Mg^{++}$–$Ca^{++}$-dependent ATPase activities were here found to be absent (fig. 1c).

*Electrophoresis.* Agarose electrophoresis was performed on fractions drawn from the gradient after centrifugation of re-suspended pellet in 20–50% sucrose. The top fraction was found to contain seminal plasma proteins (Fig. 1). The fraction with peak enzyme activities showed a protein precipitate at the application site.

The GGT staining technique was used to localize the GGT activities of specimens after agarose electrophoresis. Seminal plasma GGT gave three different mobility optima: a fast $\alpha_2$-band, a slow $\alpha_2$-band and activity at the application site. The low GGT activity of the top fraction (fraction 1 in table 1) from the supernatant was found to have fast $\alpha_2$-mobility. The considerably higher GGT activities of the middle and bottom fractions (Fractions 4 and 12 in

TABLE I. GGT activity before and after centrifugation at 100,000 × *g*, 4°C for 2 h of 6 ml pooled seminal plasma. Supernatant fractions collected with a volume of 0.5 ml each from top to bottom (Nos. 1–12). Pellet re-suspended in 0.50 ml 0.05 mol/l Tris-HCl buffer pH 7.4 with 0.15 mol/l NaCl. Protein concentration determined according to Lowry.

| | Total GGT activity (nkat) | Protein concentration (g/l) | GGT activity/ protein content (μkat/g) |
|---|---|---|---|
| Pooled seminal plasma | 750 | 41 | 3.0 |
| Supernatant fraction No. 1 | 5.5 | 39 | 0.28 |
| Supernatant fraction No. 4 | 29 | 41 | 1.4 |
| Supernatant fraction No. 8 | 37 | 40 | 1.9 |
| Supernatant fraction No. 12 | 59 | 46 | 2.5 |
| Pellet suspension | 290 | 24 | 24 |

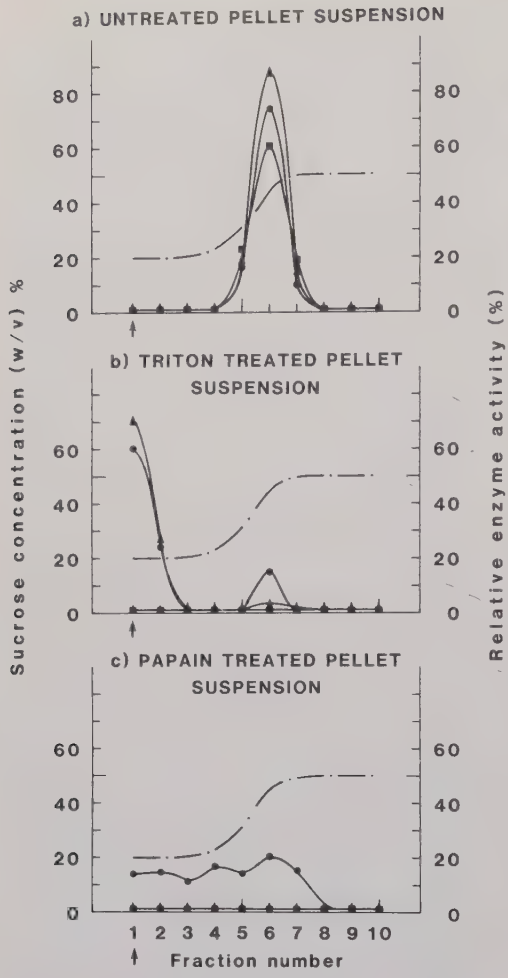


FIG. 1. Comparative distribution of GGT, ATPase and Suc(Ala)$_3$pNA-hydrolysing activities after sucrose gradient centrifugation (100,000 × $g_{av}$, 4°C, 12 h) of a) 0.20 ml seminal plasma pellet suspension; b) 0.20 ml pellet suspension treated with 1% (v/v) Triton X-100; and c) 0.20 ml pellet suspension treated with papain. One ml fractions were drawn. Fraction densities were determined with a refractometer. The contaminating seminal plasma proteins were recovered in the top fraction No. 1 (marked with arrows). (— · —) Sucrose concentration; (●) GGT activity; (▲) Suc(Ala)$_3$pNA hydrolysing activity; (■) ATPase activity.

Table I) showed slow $\alpha_2$-mobility. GGT from fraction 6 of the sucrose gradient centrifuged pellet suspension showed both slow $\alpha_2$-mobility and intense enzymatic activity at the application site (Fig. 2).

A change in pellet GGT mobility was noted after the addition of 1% Triton X-100, to the

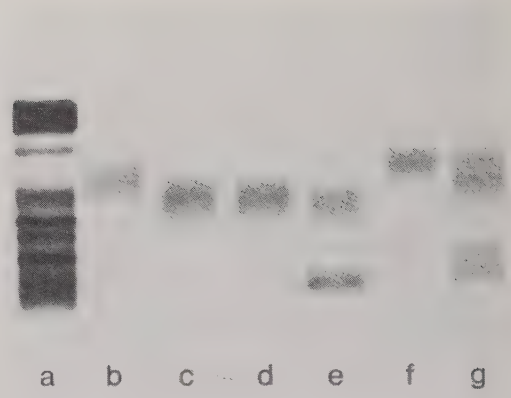


FIG. 2. Electrophoresis run in 0.075 mol/l barbital buffer pH 8.6 with 2 mmol/l calcium lactate and 1% agarose. Reference plasma was fixed in picric acid, pressed, dried, stained in Coomassie blue and washed. The samples were stained for GGT activity.
a) Reference plasma; b) Supernatant 1 from fractionated ultracentrifugation; c) Supernatant 4 from fractionated ultracentrifugation; d) Supernatant 12 from fractionated ultracentrifugation; e) Re-suspended pellet from ultracentrifugation of seminal plasma; f) Fraction 1 from sucrose gradient centrifugation of papain treated pellet suspension; and g) Fraction 6 from sucrose gradient centrifugation of papain treated pellet suspension.

agarose gel and samples. The GGT activity was then found to be of slow $\alpha_2$-mobility, and no enzymatic activity was seen at the application site.

Top fractions from sucrose gradient centrifugation of papain treated pellet suspension showed GGT activity with the fast $\alpha_2$-mobility mentioned above (Fig. 2).

Charge shift electrophoresis was performed with addition of the anionic detergent sodium deoxycholate and the cationic detergent cetyltrimetylammoniumbromide (CTAB) to agarose gels and to samples containing non-ionic detergent. The water-solubility of the GGT with the fast $\alpha_2$-mobility, from the top fraction of the supernatant, was confirmed by the fact that neither it nor the papain treated pellet showed any electrophoretic shift after the addition of sodium deoxycholate or of CTAB. The detergent binding properties of the GGT with the slow $\alpha_2$-mobility, from the bottom fractions of the supernatant, were demonstrated by the fact that both it and the untreated pellet suspension showed an anodal shift following the addition of sodium deoxycholate, and a cathodal shift following the addition of CTAB.

Seminal plasma from the subject with pure prostatic secretion was analysed by agarose electrophoresis after ultracentrifugation of the sample. After re-suspension part of the pellet obtained was treated with papain. The water-soluble form of GGT with fast $\alpha_2$-mobility was found both in the top fraction of the supernatant and in the papain treated pellet. Untreated pellet showed enzyme activity at the application site and with slow $\alpha_2$-mobility.

## DISCUSSION

Subcellular organelles of prostatic origin, with a density of 1.03 g/ml and a diameter of 20–150 nm, have been found to be carriers of a $Mg^{++}$–$Ca^{++}$-dependent ATPase [15], endogenous protein kinase activity [19], and a $Zn^{++}$-dependent peptidase hydrolysing $Suc(Ala)_3pNA$ [11]. These subcellular organelles were here shown to contain the integral membrane enzyme GGT. This conclusion was based on the presence in the same fraction of GGT, $Zn^{++}$-dependent peptidase, and $Mg^{++}$–$Ca^{++}$-dependent ATPase activities, after gradient centrifugation of pellet from seminal plasma, and on the distinct peak of GGT activity at a density of 1.03 g/ml.

The size range of the organelles does not permit penetration into 1% agarose gels. Once the organelle-bound enzymes GGT and $Zn^{++}$-dependent peptidase had been rendered soluble in active form with the non-ionic detergent, Triton X-100, the small GGT micelles thus formed were able to penetrate agarose gels. After the organelles had been treated with papain, most of the GGT was released in an active form. The molecular difference between this released, water-soluble form of GGT and the organelle-bound form might be similar to that found in the rat kidney enzyme, where a hydrophobic domain constitutes the N-terminal part of the heavy chain of the enzyme that anchors GGT to the membrane. This hydrophobic domain is cleaved off by proteolytic enzymes such as papain [20]. The loss in activity of the organelle-bound $Zn^{++}$-dependent peptidase, and of the $Mg^{++}$–$Ca^{++}$-dependent ATPase, after digestion by papain, and the absence of released peptidase and of ATPase activities, all suggest cleavage near the active site. The presence of the water-soluble form of GGT, in both pure prostatic secretion and seminal plasma, implies that a proteolytic enzyme in prostatic fluid may be digesting the membrane anchor of GGT, analogous to the papain effect. The origin of GGT with detergent binding properties, but not bound to the subcellular organelles, will be the subject of further investigation.

The function of the very high GGT activity in human seminal fluid remains to be discovered. Prostatic secretion has a pH of approximately 6.5. At this pH, both GGT- and $Zn^{++}$-dependent peptidase activities are very low. As secretions from the accessory sex glands mingle, however, the pH rises to just below 8, which is optimal for both GGT- and $Zn^{++}$-dependent peptidase. This would seem to indicate a specific function in the seminal fluid for these enzymes, and for the organelles that carry them.

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# Synthetic protease inhibitors and post-ejaculatory degradation of human semen proteins

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Lilja, H. &. Weiber, H. Synthetic protease inhibitors and post-ejaculatory degradation of human semen proteins. *Scand. J. clin. Lab. Invest.* **44**, 433–438, 1984.

Normal post-ejaculatory proteolytic changes in human seminal plasma rapidly distort its electrophoretic protein pattern. This invalidates the electrophoretic evaluation of the content in the secretion from the accessory sex glands. Both agarose and sodium dodecylsulphate polyacrylamide gel electrophoresis (SDS-PAGE) were used to study the proteolytic changes and how they could be modified by various synthetic protease inhibitors. Liquefaction of coagulated semen could be inhibited by adding *o*-phenanthroline directly after ejaculation, whereas neither neutrally buffered $Na_2EDTA$, di-isopropylfluorophosphate (DFP), benzamidine, nor thiol reagents proved effective. Addition of the serine protease inhibitors DFP and benzamidine, *o*-phenanthroline, and iodoacetamide substantially retarded the proteolytic alterations of the proteins as demonstrated by both agarose electrophoresis and SDS-PAGE. We recommend that electrophoretic protein analysis of human semen be performed on ejaculates collected in vessels containing protease inhibitors. For routine analysis, the addition of benzamidine ensures sufficiently stable proteins to permit reliable electrophoretic analysis of samples stored at room temperature for 4 h.

*Key-words:* prostate; protein degradation; protease inhibitors; semen; seminal vesicles

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The proteins occurring in human seminal plasma are mainly secreted by the seminal vesicles and the prostate gland, about 55–60% originating from the seminal vesicles and 35–40% from the prostate [6]. The prostate contributes acidic proteins that can be electrophoretically separated on agarose from the bulk of basic proteins originating from the seminal vesicles [7]. On ejaculation, secretions from the accessory sex glands are released sequentially and mix with spermatozoa [12]. Ejaculated semen immediately forms a coagulum which, it has been proposed, is made up of proteins from the seminal vesicles [11]. The prostatic secretion is rich in proteolytic enzymes and is responsible for liquefaction of the coagulum [11], lysis normally being complete within 20 min of ejaculation [15]. Two proteases, a neutral protease [13] and a 'collagenase-like' peptidase [8], have been proposed as being involved in this process [8, 13, 14]. The occurrence, after liquefaction, of further proteolytic changes in seminal plasma proteins has been reviewed by Mann [10]. It is reflected in progressive alterations of the electrophoretic

protein pattern and in an increasing concentration of free amino acids, though the proteases involved are poorly characterized.

Any evaluation of native proteins secreted by the seminal vesicles and the prostate gland necessitates inhibition of post-ejaculatory proteolysis in human semen. This study deals with proteolytic alterations in semen, and how the addition of synthetic protease inhibitors affects the stability of the proteins. We present a semen sampling procedure ensuring sufficiently stable proteins to permit reliable electrophoretic evaluation of the seminal vesicle and prostatic contribution.

## MATERIAL AND METHODS

*Chemicals.* Benzamidine chloride and *o*-phenanthroline-HCl were obtained from E. Merck (Darmstadt, FGR); di-isopropylfluorophosphate (DFP) from Fluka A.G. (Buchs, Switzerland); iodoacetamide, 5,5-dithiobis-2-nitrobenzoic acid (DTNB) and L-cysteine-HCl from Sigma Chemical Company (St Louis, Mo., USA); and acrylamide (electrophoresis grade), N-N-methylene-bisacrylamide from British Drug Houses (Poole, England). Molecular weight standards were obtained from Pharmacia Fine Chemicals (Uppsala, Sweden), and Seakem® agarose from FMC Corp., Marine Colloids Div. (Rockland, Me., USA). Beta$_2$-microglobulin was available at the laboratory. All other chemicals used were of reagent grade.

*Sample collection.* Human semen, from patients undergoing investigation for involuntary infertility or from voluntary donors, was obtained from the fertility laboratories in Malmö, Sweden, all specimens having been collected by masturbation. To study the post-ejaculatory protein degradation the samples were at first allowed to liquefy at room temperature. Within 5 min of liquefaction, in certain cases after the addition of synthetic protease inhibitors, the liquefied semen was centrifuged at 800 *g* for 10 min and seminal plasma was obtained as the separated supernatant. The seminal plasma was either used immediately or stored frozen at −20°C until use.

To study the liquefaction process, coagulated semen with an estimated normal liquefaction rate was collected in vessels that were placed in ice-cold $H_2O$ immediately after ejaculation. The coagulated samples were then briefly cyclomixed, and in certain cases protease inhibitors were added to portions of the ejaculate followed by a brief cyclomixing, and then either used immediately or frozen at −70°C until use.

*Chemical analyses.* Fructose was measured according to de Carvalho & Pogell [2], albumin by electroimmunoassay and zinc by atomic absorption spectrometry.

*Electrophoretic techniques.* Agarose gel electrophoresis, at pH 8.6, was performed according to standard methods [5]. Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) in a 12–18% (w/v) gel gradient was done as described by Maizel [9], and by Blobel & Dobberstein [1]. Reduced samples with a volume of 80 µl (having an estimated protein content of 10–15 µg) were applied to the spacer gel. The gels were run at constant current (25 mA) for 16–20 h.

*Estimation of liquefaction of coagulated semen.* These studies were performed at 37°C, samples being slowly rocked, and liquefaction estimated to have occurred when gel-clumps were no longer observed and the fluid semen could be drawn into a Pasteur pipette.

*General experimental procedures.* To study degradation of semen proteins, the samples were stored at room temperature or 4°C for 0 to 24 h. Samples containing added exogenous protease inhibitors were not usually diluted more than 10% with a stock solution of a protease inhibitor. The following stock solutions were used: 1 mol/l benzamidine-HCl, 1 mol/l DFP, 0.3 mol/l $Na_2EDTA$ buffered to pH 7.4, 0.5 mol/l *o*-phenanthroline-HCl, 20 mmol/l DTNB in 0.1 mol/l sodium phosphate pH 7.4 and 0.3 mol/l iodoacetamide buffered to pH 7.4. All electrophoretic experiments were made on seminal plasma from a minimum of 10 different subjects. Each sample was divided in 50–100 µl portions, to which the different protease inhibitors were then added. The spontaneously sedimented precipitate in seminal plasma, stored in test tubes (10 ml) with a conical bottom, was estimated by visual inspection.

## RESULTS

### *I. Liquefaction of coagulated semen*

Coagulated semen liquefied completely within

20 min at 37°C ($n = 5$). Portions from these samples (approximately 0.5 ml each) were used for the estimation of liquefaction in the presence of exogenous protease inhibitors. *O*-phenanthroline-HCl, at 50 mmol/l, totally inhibited liquefaction of coagulated semen. At an *o*-phenanthroline concentration below 40 mmol/l, complete liquefaction of semen occurred. Semen liquefied completely within 20 min in the presence of 30 mmol/l neutrally buffered $Na_2EDTA$, 10 mmol/l DFP, 100 mmol/l benzamidine, or 30 mmol/l iodoacetamide. Coagulated semen that was acidified to approximately pH 5 (with acetic acid) liquefied completely at 37°C within 20 min ($n = 4$).

*II. Post-ejaculatory degradation of seminal plasma proteins*

*1. Degradation in the absence of exogenous synthetic protease inhibitors.* The electrophoretic protein pattern of the supernatant of coagulated semen (after centrifugation at 3000 *g* for 2 min) was found not to be representative of liquefied semen (Figs 1a, b). During liquefaction of semen several basic proteins appeared. When seminal plasma was stored at room temperature after the liquefaction for 0 to 24 h, alterations in the protein pattern appeared within the first hour. The electrophoretic protein pattern was severely distorted after more than 4 h storage. Storage at

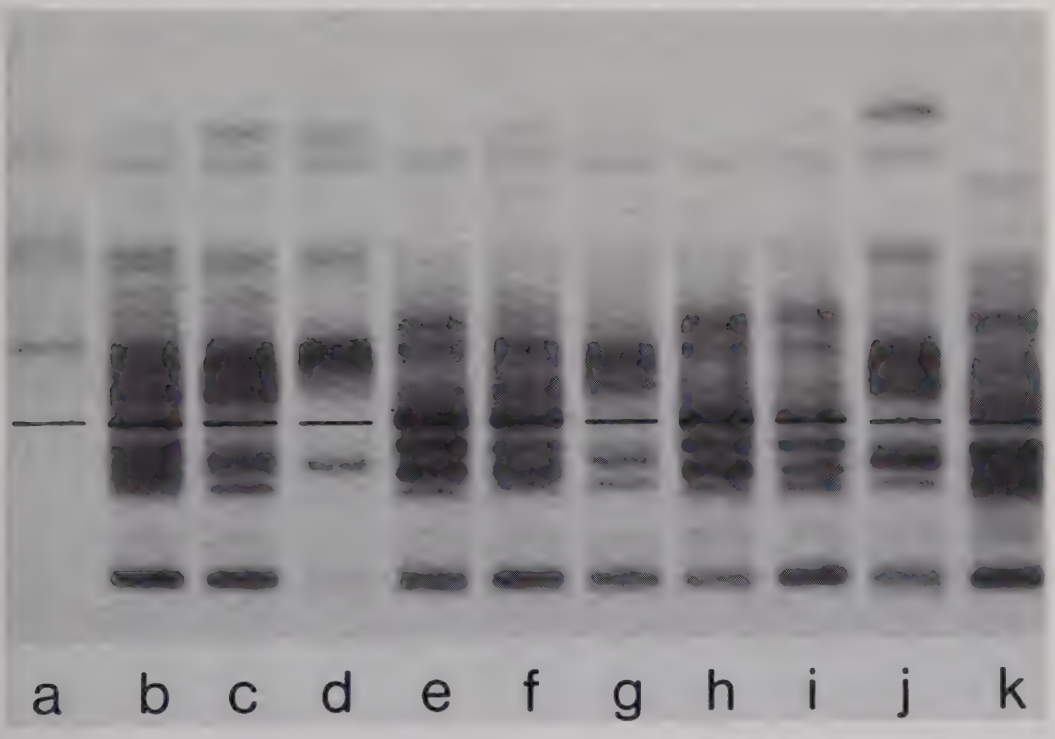


FIG. 1. Agarose electrophoresis, at pH 8.6, of human semen from one subject after storage at room temperature for 0 to 24 h after ejaculation. The sample was divided into portions to which, in certain cases, protease inhibitors were added before or directly after liquefaction of semen. Anode at top. (a) Supernatant of coagulated semen after centrifugation at 3000 *g* for 2 min. (b) Liquefied seminal plasma, analysed within 0.5 h from ejaculation. (c) Seminal plasma 4 h after liquefaction. (d) Seminal plasma 24 h after liquefaction. (e) Seminal plasma liquefied in the presence of benzamidine, 100 mmol/l, analysed within 0.5 h from ejaculation. (f) Seminal plasma liquefied in the presence of benzamidine, 100 mmol/l, 4 h after liquefaction. (g) Seminal plasma liquefied in the presence of benzamidine, 100 mmol/l, 24 h after liquefaction. (h) DFP, 10 mmol/l, added to liquefied seminal plasma. Sample stored for 24 h after liquefaction. (i) *O*-phenanthroline-HCl, 50 mmol/l, added to liquefied seminal plasma. Sample stored for 24 h after liquefaction. (j) Neutrally buffered $Na_2EDTA$, 50 mmol/l, added to liquefied seminal plasma. Sample stored for 24 h after liquefaction. (k) Iodoacetamide, 30 mmol/l, added to liquefied seminal plasma. Sample stored for 24 h after liquefaction.

4°C slightly delayed the appearance of the electrophoretic protein pattern alterations (not shown). The most apparent change noted after storage for 24 h of seminal plasma was the disappearance of basic proteins having an estimated molecular weight below 18,000 (Figs 1d, 2d).

*2. Degradation in the presence of serine protease inhibitors.* Semen that liquefied in the presence of benzamidine (100 mmol/l) or DFP (10 mmol/l) had an altered protein pattern on agarose electrophoresis (Fig. 1e), or on SDS-PAGE (Fig. 2e), as compared with semen liquefied in the absence of exogenous protease inhibitor (Figs 1b, 2b). Addition of DFP or benzamidine to semen directly after liquefaction

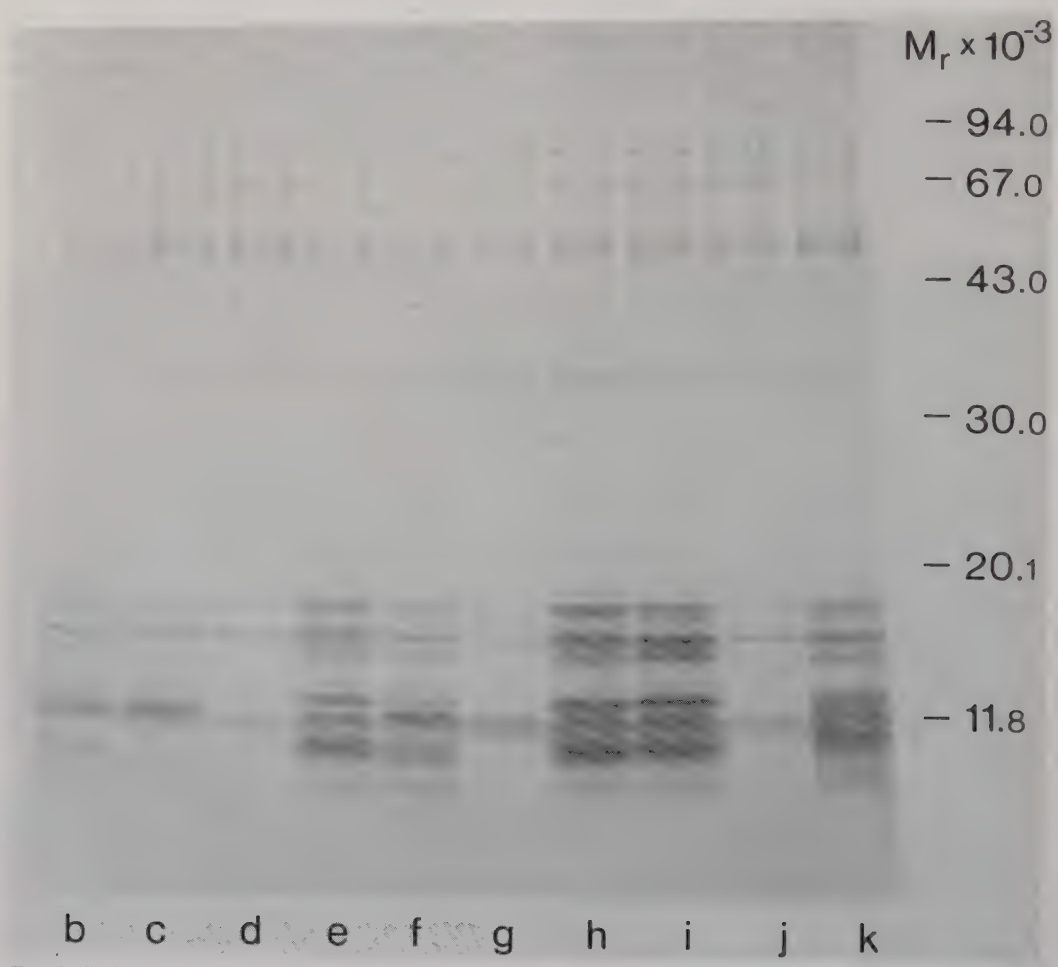


FIG. 2. SDS-PAGE of human semen from one subject after storage at room temperature for 0 to 24 h after ejaculation. The sample was divided into portions to which, in certain cases, protease inhibitors were added before or directly after liquefaction of semen. The molecular weight marker proteins used were phosphorylase B (94,000); albumin (67,000); ovalbumin (43,000); carbonic anhydrase (30,000); soybean trypsin inhibitor (20,100); and $\beta_2$-microglobulin (11,800). (b) Liquefied seminal plasma, analyzed within 0.5 h from ejaculation. (c) Seminal plasma 4 h after liquefaction. (d) Seminal plasma 24 h after liquefaction. (e) Seminal plasma liquefied in the presence of benzamidine, 100 mmol/l, analysed within 0.5 h from ejaculation. (f) Seminal plasma liquefied in the presence of benzamidine, 100 mmol/l, 4 h after liquefaction. (g) Seminal plasma liquefied in the presence of benzamidine, 100 mmol/l, 24 h after liquefaction. (h) DFP, 10 mmol/l, added to liquefied seminal plasma. Sample stored for 24 h after liquefaction. (i) *O*-phenanthroline-HCl, 50 mmol/l, added to liquefied seminal plasma. Sample stored for 24 h after liquefaction. (j) Neutrally buffered $Na_2EDTA$, 50 mmol/l, added to liquefied seminal plasma. Sample stored for 24 h after liquefaction. (k) Iodoacetamide, 30 mmol/l, added to liquefied seminal plasma. Sample stored for 24 h after liquefaction.

produced no immediate changes of the protein pattern in these samples (not shown). Seminal plasma containing 10 mmol/l DFP retained the 'original' protein pattern of newly liquefied semen even after storage for 24 h at room temperature (Figs 1h, 2h). Samples that contained 100 mmol/l benzamidine did not retain the 'original' protein pattern after storage at room temperature for 24 h, but were only partially degraded (Figs 1g, 2g). After storage of these samples for 4 h, the pattern on both agarose electrophoresis (Fig. 1f) and SDS-PAGE (Fig. 2f) was comparable with that of semen shortly after liquefaction in the absence of any exogenous protease inhibitor (Figs 1b, 2b).

*3. Degradation in the presence of metal chelating compounds.* Addition of neutrally buffered $Na_2EDTA$ or *o*-phenanthroline to liquefied semen changed the position of some protein bands on agarose electrophoresis. These bands, having a mobility close to that of albumin, seemed to migrate faster in the presence of metal chelating compounds. Apart from this immediate change, the protein pattern remained unchanged after storage for 24 h of seminal plasma containing 50 mmol/l of *o*-phenanthroline (Figs 1i, 2i). At an *o*-phenanthroline concentraton of 10 mmol/l, no retardation of the normal protein degradation of semen was observed (not shown). The protein degradation of seminal plasma containing 50 mmol/l of neutrally buffered $Na_2EDTA$ was partially retarded but after sample storage for 24 h the protein pattern was severely distorted (Figs 1j, 2j).

*4. Degradation in the presence of thiol reagents.* Addition of iodoacetamide to liquefied semen changed the mobility of albumin on agarose electrophoresis. Apart from this, the protein pattern remained almost undegraded after 24-h storage of seminal plasma containing 30 mmol/l iodoacetamide (Figs 1k, 2k). DTNB, at 10 mmol/l concentration in semen, was less effective in the prevention of protein degradation than iodoacetamide at 30 mmol/l.

### *III. Other effects of adding synthetic protease inhibitors to semen*

The amount of precipitate formed in 5 ml of seminal plasma, pooled from 10 subjects, was estimated to be more than 50% reduced after storage for 24 h in samples containing iodoacetamide (30 mmol/l), as compared with the reference (containing no exogeneous protease inhibitor). This is consistent with prostatic phosphatase being inhibited by sulph-hydryl binding substances [3], resulting in diminished release of phosphate capable of reacting with spermine [11].

Spermatozoa were immobilized by benzamidine-HCl (100 mmol/l) as well as by the addition of *o*-phenanthroline-HCl (50 mmol/l) to semen.

The concentrations of zinc, fructose, and albumin were measured in 34 seminal plasma samples, each divided into two separate portions. Benzamidine (100 mmol/l) was added to one of the portions and the ratios between measurement in absence vs presence of benzamidine were calculated. The mean ratios were for fructose $\bar{x} = 1.05 \pm 0.07$; for zinc $\bar{x} = 0.998 \pm 0.05$, and for albumin $\bar{x} = 1.01 \pm 0.08$ ($n = 34$).

## DISCUSSION

The soluble proteins in coagulated semen were found not to be representative of the proteins secreted from the accessory sex glands. During liquefaction several basic proteins were released into the fluid phase of semen. The clot liquefaction was further inhibited by *o*-phenanthroline-HCl, an inhibition that was concentration-dependent but not dependent on the acidic pH (5 to 6) obtained after *o*-phenanthroline addition. The protease(s) responsible for liquefaction can thus be inhibited by one metal-chelating compound but, according to our results and those of Lukač & Koren [8], clot lysis is not inhibited by $Na_2EDTA$. Neither serine protease inhibitors nor iodoacetamide inhibited liquefaction. These results on semen liquefaction are inappropriate to a discussion of the proposed participation of the collagenase-like peptidase [8] and of the neutral protease seminin [13, 14] in the lysis reaction. However, by adding *o*-phenanthroline to coagulated semen directly after ejaculation, we have evolved a way of studying the native protein composition of semen before liquefaction.

DFP was the most efficient inhibitor of the rapid post-liquefaction degradations of seminal plasma proteins, which indicates that serine protease(s) is(are) mainly responsible for this process. *O*-phenanthroline or iodoacetamide

inhibited the degradation of proteins in liquefied semen almost as effectively. The post-lytic proteolytic alterations of semen proteins were minimized by collecting the ejaculate in vessels containing various protease inhibitors that did not affect the clot liquefaction. It has previously been shown that the disappearance of some basic proteins from seminal plasma was retarded by a combination of phenylmethyl-sulphonylfluoride (PMSF) and EDTA [4]. Owing to their toxicity, neither DFP, PMSF nor iodoacetamide is to be recommended for the routine sampling of semen. The addition of the serine protease inhibitor benzamidine to semen retarded seminal plasma protein degradation more efficiently than did neutralized $Na_2EDTA$. The protein alterations that occurred in semen containing benzamidine (0.1 mol/l) were insignificant during storage of samples for the first 4 h. Benzamidine did not interfere with the determination of zinc, fructose or albumin.

We recommend that seminal plasma protein analysis be performed in samples where the ejaculate is collected in a vessel containing benzamidine-HCl (e.g. 0.7 ml of a 1 mol/l solution), giving a final semen concentration of benzamidine of 0.1 mol/l or higher. In such ejaculates the electrophoretic protein pattern is reproducible for 4 h, even when semen has been stored at room temperature. The evaluation of spermatozoa variables should be done in ejaculates that do not contain benzamidine as this compound affects the mobility of the spermatozoa.

## ACKNOWLEDGMENTS

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# Characterization of the predominant basic protein in human seminal plasma, one cleavage product of the major seminal vesicle protein

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Lilja, H., Laurell, C.-B. & Jeppsson, J.O. Characterization of the predominant basic protein in human seminal plasma, one cleavage product of the major seminal vesicle protein. *Scand. J. clin. Lab. Invest.* **44**, 439–446, 1984.

From liquefied human seminal plasma, we purified the predominant basic protein which appears following liquefaction of coagulated semen. The protein was purified in the presence of di-isopropylfluorophosphate to retard its degradation. Heparin-Sepharose® chromatography was followed by gel filtration (Biogel® P 60) and by fast performance liquid chromatography on a reversed phase column ($C_8$). The basic protein is a single polypeptide chain with an apparent molecular mass of 12.8 kDa, and has a pI value between that of trypsinogen (9.3) and cytochrome C (10.3). The protein contains no carbohydrate, is rich in histidine, glutamate, and lysine, but is devoid of both cysteine and methionine. The amino-terminal portion of the protein sequence is unique: H N K Q E G R D H D K S K G H F H R V V I H H K G G K A H R G -. A specific rabbit antiserum was raised against the 12.8 kDa basic protein. The protein was found to be unique to seminal plasma among all extracellular fluids examined. Three immunologically related 52 kDa, 71 kDa, and 76 kDa proteins were identified in seminal vesicle secretion when it had been reduced. Prostatic enzyme(s) degraded these proteins to the 12.8 kDa basic protein and several other basic proteins with apparent molecular masses below 18 kDa.

*Key-words:* peptide fragments; protease inhibitors; prostate; semen; seminal vesicles

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Human seminal plasma is a protein rich fluid (35–50 g/l), in which the proteins readily undergo proteolysis [17]. A number of blood plasma proteins are present in seminal plasma at low concentrations [21]. The majority of seminal plasma proteins are secreted by the accessory sex glands. Proteins originating from the prostate can be partially separated from the bulk of basic seminal vesicle proteins by agarose electrophoresis [14]. The prostatic secretion is rich in proteolytic enzymes. Proteins derived from the seminal vesicles predominate quantitatively over those secreted by the prostate gland [21]. The function of the vesicular proteins is unknown but it has been suggested that they constitute the structural skeleton of coagulated semen [17], and some seminal vesicle proteins have been identified as sperm coating antigens [9].

Care must be taken to prevent degradation of semen proteins during purification, because of the proteolytic activity of seminal plasma even at 4°C. We reported recently on the post-ejaculatory degradation of seminal plasma proteins and the effects of adding various synthetic protease inhibitors, serine protease inhibitors being shown to inhibit the degradation of the predominating basic protein in liquefied seminal plasma [13]. This basic protein appears following the liquefaction of coagulated semen [13], and here we describe the purification of this protein and some of its characteristics.

## MATERIAL AND METHODS

*Chemicals.* Heparin-Sepharose®, pI determination kit, and molecular weight markers for polyacrylamide gel electrophoresis and gel filtration were obtained from Pharmacia Fine Chemicals (Uppsala, Sweden); Ultropac® TSK G 2000 SW, and Ampholines® from LKB (Bromma, Sweden); Biogel® P 60 from Bio-Rad Laboratories (Richmond, Calif., USA); Cytochrome C (from horse heart), myoglobin (from equine skeletal muscle), di-isopropyl-fluorophosphate (DFP), benzamidine-HCl, and 3-amino-9-ethyl-carbazole from Sigma Chemical Co. (St Louis, Mo., USA); Isogel® and Seakem® agaroses from FMC Corp., Marine Colloids Div. (Rockland, Me., USA); and acrylamide (electrophoresis grade) and N′ N methylenebisacrylamide from British Drug Houses (Poole, UK). Beta$_2$-microglobulin and peroxidase coupled goat anti-rabbit IgG were available at the laboratory. All other chemicals used were of reagent grade.

*Sample material.* Human semen, from patients undergoing investigation for involuntary infertility or from voluntary donors, was obtained from the fertility laboratories in Malmö, Sweden, all specimens having been collected by masturbation. The samples were allowed to liquefy at room temperature. Seminal plasma was obtained as the separated supernatant after centrifugation of the samples at 800 *g* for 15 min. The seminal plasma was frozen at −20°C within 3 h of collection and stored frozen until used.

Pure prostatic secretion was collected from a subject with aplasia of the seminal vesicles and the deferent ducts, as verified by computer tomography and semen analysis. Seminal vesicle secretion, collected post-mortem, from three men, 39, 65, and 66 years old, was sampled at autopsy within 12 h of death and frozen at −70°C until used.

*Electrophoretic techniques.* Agarose electrophoresis and immunoelectrophoresis were performed according to standard procedures [11]. Isoelectric focusing in polyacrylamide gels was performed in a pH 7–11 gradient in an atmosphere of nitrogen, according to standard procedures [10]. Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE), in a 10–15% or 12–18% (w/v) gel gradient was done as described by Maizel [18] and Blobel & Dobberstein [1]. The gels were run at constant current (20 mA) for 16 h. Reduced samples with a volume of 80 µl, having an estimated protein content of 15–20 µg, were applied to the gels. Immunologic identification after SDS-PAGE was made with the Western blotting technique described by Burnette [2], and immunodetection after agarose electrophoresis was done essentially as described by Glynn *et al.* [5]. Immune complexes were detected with horseradish peroxidase coupled goat anti-rabbit IgG, and were visualized with 3-amino-9-ethyl-carbazole according to Graham *et al.* [6].

*Purification of the predominant basic seminal plasma protein.* All chromatography, dialysis, and concentration steps were performed at 4°C, if not otherwise stated. Diaflo® membranes with a cut off range of 2 kDa and dialysis tubing (Spectropore®) with a cut off range of 3.5 kDa were used throughout. After chromatography, the basic protein was detected in individual fractions by agarose electrophoresis, in which it migrated as the major, clearly demarcated most basic protein (Fig. 1a). Seminal plasma samples, containing a high concentration of the basic protein as estimated by agarose electrophoresis, were pooled to 50 ml, and 0.5 ml of 1 mol/l DFP was added to the pool directly after thawing. The pool was centrifuged at 15,000 *g* for 15 min at 4°C, and the supernatant applied on a heparin-Sepharose column (140 × 23 mm) previously equilibrated with 50 mmol/l Tris, pH 8.0, 0.15 mol/l NaCl and 10 mmol/l benzamidine. The column was eluted with a linear 0.15–1.5 mol/l NaCl-gradient (200 + 200 ml). The basic protein eluted at an NaCl concentration ranging from 0.8 to 1.0 mol/l. The fractions were pooled, concentrated on a Diaflo membrane and dialysed against

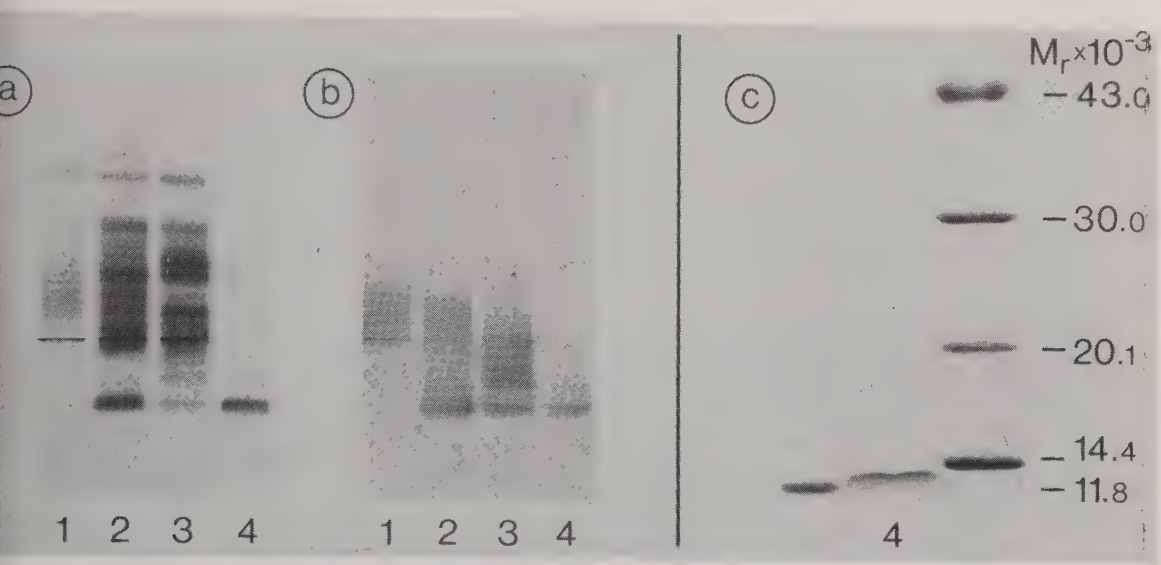


FIG. 1a. Electrophoresis in low endosmosis agarose (10 g/l) at pH 8.6. Anode at top. 1, Seminal vesicle secretion; 2, seminal plasma two hours after liquefaction; 3, seminal plasma liquefied in presence of 10 mmol/l of DFP; 4, purified basic protein. (b) Immunodetection with antiserum raised against the purified basic protein after agarose electrophoresis. Anode at top. 1, Seminal vesicle secretion; 2, seminal plasma 2 h after liquefaction; 3, seminal plasma liquefied in presence of 10 mmol/l DFP; 4, purified basic protein. (c) SDS-PAGE in a 12–18% (w/v) gel gradient. The molecular weight marker proteins used were ovalbumin (43,000); carbonic anhydrase (30,000); soybean trypsin inhibitor (20,100); $\alpha$-lactalbumin (14,400); and $\beta_2$-microglobulin (11,800). 4, Purified basic protein.

50 mmol/l Tris, pH 8.0, with 0.4 mol/l of NaCl and 10 mmol/l of benzamidine. After adding DFP to the pool to a final concentration of 10 mmol/l, it was applied on top of a Biogel P 60 column (900 × 16 mm) that had been equilibrated in the same buffer. The column was eluted at a flow rate of 8.9 ml/h and fractions containing the basic protein were pooled and concentrated on Diaflo. Some of this material was used for immunization procedures described below. DFP was again added to the remaining part of the pool to a final concentration of 10 mmol/l, which was dialysed against 50 mmol/l $NH_4HCO_3$, pH 8.2, and then evaporated under vacuum or lyophilized. Portions of this material, with an approximate protein content of 1 mg, were dissolved in 26 mmol/l trifluoroacetate also containing 0.95 mol/l of acetonitrile. Final purification of this material was performed with reversed phase chromatography on a Pharmacia FPLC® system equipped with a Pharmacia HR 5/10 Pro RPC prepacked column ($C_1/C_8$) that had been equilibrated with the same buffer. Elution was performed, at room temperature, with a linear gradient against 11.4 mol/l acetonitrile at a flow rate of 0.2 ml/min (Fig. 2a).

*Molecular weight estimation with HPLC gel filtration.* Purified basic protein, obtained as described above, and molecular weight marker proteins were dissolved in 0.1 mol/l ammonium acetate, pH 5.5, and chromatographed at room temperature on an Ultropac TSK G 2000 column (600 × 7.5 mm) that was equilibrated in the same buffer (Fig. 2b).

*Raising of a specific antiserum against the basic protein.* Basic protein, purified as above, was coupled with bovine serum albumin (BSA) using glutaraldehyde as a coupling reagent [22]. A BSA to basic protein molar ratio of 1:2 was used. Rabbits were immunized with 0.5 mg of the BSA-coupled basic protein emulsified with complete Freund's adjuvant. The rabbits were boostered continuously, at 4-week intervals, and regularly bled. The antiserum was depleted of anti-albumin IgG by being passed through an albumin-coupled Sepharose column prepared according to standard methods.

*Amino acid analysis.* Purified basic protein

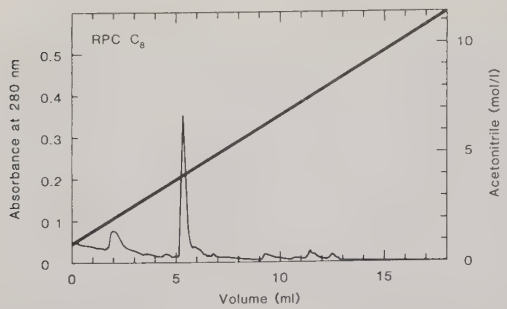


FIG. 2a. FPLC reversed-phase chromatography on a $C_8$ column of partially purified basic protein obtained from gel filtration. The solid line indicates the acetonitrile elution gradient (from 0.95 to 11.4 mol/l of acetonitrile).

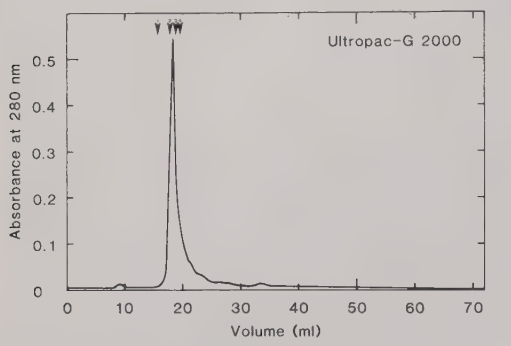


FIG. 2b. HPLC gel filtration on an Ultropac TSK G 2000 column of the purified basic protein. The molecular weight marker proteins used were 1, ovalbumin (43,000); 2, myoglobin (17,000); 3, ribonuclease A (13,700); and 4, cytochrome C (12,800).

was reduced for 1 h in 0.5 mol/l Tris, pH 8, with 6 mol/l guanidin-HCl and 25 mmol/l dithiothreitol and then carboxymethylated with 50 mmol/l iodoacetic acid for 2 h under darkness. Two-hundred-fifty-microgram portions of the carboxymethylated protein were hydrolysed with 6 mol/l HCl in evacuated sealed tubes at 110°, for 24 and 72 h. The dried hydrolysates were analysed on a Beckman 119 CL amino acid analyser. The tryptophan content of the protein was calculated from the UV absorbance spectrum in 6 mol/l guanidin-HCl according to Edelhoch [3].

*Sequence determination.* Sequence determination of the $NH_2$-terminal portion of the purified basic protein was made by automatic Edman degradations in a Beckman 890 C liquid phase sequencer, using 1.0 mol/l Quadrol and polybrene as a protein carrier. PTH amino acids were identified by HPLC on a μBondapak® $C_{18}$ reverse phase column (Waters). The elution system used was an 11–38% (v/v) ethanol gradient in 0.5 mmol/l sodium acetate pH 5.1 [4].

## RESULTS

### *I. Comments on the purification of the predominant, basic protein in seminal plasma*

The majority of the anionic seminal plasma proteins passed through the heparin-Sepharose column at an NaCl concentration of 0.15 mol/l, the basic protein eluting at 0.8–1.0 mol/l of NaCl. The subsequent gel filtration on Biogel P 60 separated the basic protein from contaminants eluting in the void volume, but the protein eluted indistinctly and in a bimodal peak. Pooled basic protein from either of these peaks had, however, the same estimated molecular mass on SDS-PAGE with or without prior reduction of the samples. A recovery of about 80% after heparin-Sepharose, and a total recovery after gel filtration of about 55%, was calculated from scanning densitometry after agarose electrophoresis of the different pools. In the final purification step on the FPLC $C_8$ reversed phase column, more than 80% of the applied material eluted as a homogenous peak, identified as the basic protein (Fig. 2a).

### *II. Determination of molecular weight and pI of the basic protein*

The purified basic protein was gel filtrated on an Ultropac G 2000 column from which it eluted as a single peak with a retention corresponding to a molecular mass of 15 kDa (Fig. 2b). On SDS-PAGE the reduced and unreduced protein appeared as a single band with an apparent molecular mass of 12.8 kDa (Fig. 1c). On isoelectric focusing and agarose gel electrophoresis at pH 8.6, the basic protein migrated between trypsinogen (pI 9.3) and cytochrome C (pI 10.3).

### *III. Specificity of antiserum raised against the purified basic protein*

The raised rabbit antiserum identified the purified basic protein after SDS-PAGE (Fig. 3c),

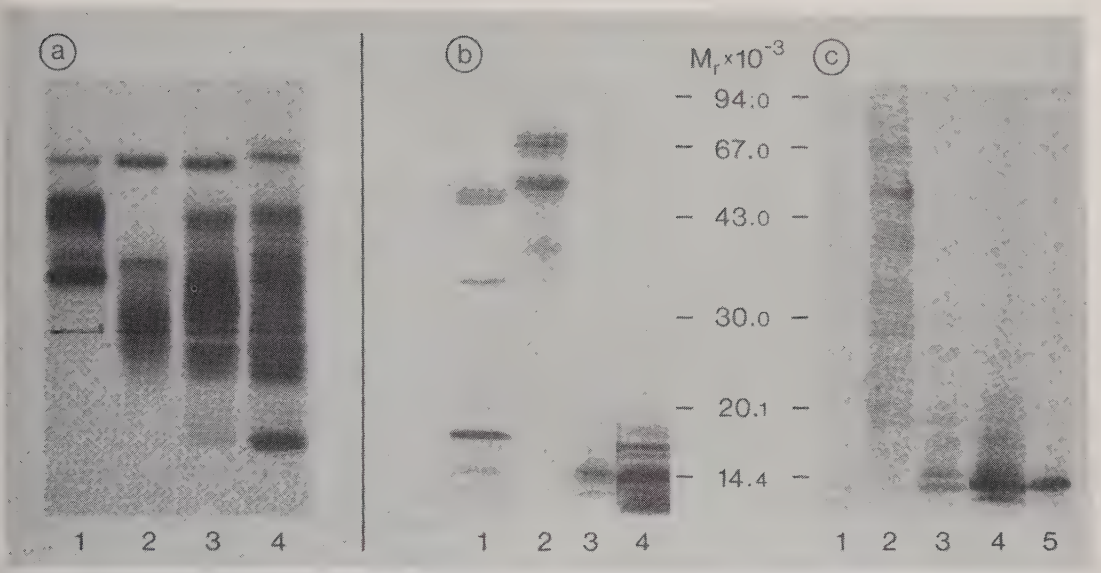


FIG. 3a. Agarose electrophoresis at pH 8.6. Anode at top. 1, Prostatic secretion; 2, seminal vesicle secretion; 3, seminal vesicle secretion (20 μl) that was incubated for 30 min at 37°C, with prostatic secretion (10 μl) before application; 4, seminal plasma liquefied in the absence of any protease inhibitor. (b) SDS-PAGE in a 10–15% (w/v) gel gradient. About 15–20 μg of protein was applicated to each lane. 1, Prostatic secretion; 2, seminal vesicle secretion; 3, seminal vesicle secretion (20 μl) that was incubated, for 30 min at 37°C, with prostatic secretion (10 μl) before application; 4, seminal plasma liquefied in the absence of any protease inhibitor. (c) Immunodetection with antiserum raised against the purified basic protein after SDS-PAGE. About 1–2 μg of protein was applicated to each lane. 1, Prostatic secretion; 2, seminal vesicle secretion; 3, seminal vesicle secretion (20 μl) that was incubated for 30 min at 37°C, with prostatic secretion (10 μl) before application; 4, seminal plasma liquefied in absence of any protease inhibitor; 5, purified basic protein.

or after agarose electrophoresis in low endosmosis agarose (Fig. 1b). The antiserum gave a single precipitation arc with the purified basic protein on immunoelectrophoresis, but on analysis of seminal plasma and seminal vesicle secretion undulating precipitation lines with non-similar appearance were obtained. Immunoelectrophoresis of blood plasma, urine, breast milk or saliva gave no precipitation arcs with this antiserum.

A small portion of rabbit antiserum was adsorbed with purified basic protein. On immunoelectrophoresis this adsorbed antiserum gave no precipitate lines against the purified basic protein, seminal plasma or seminal vesicle secretion. No proteins could be identified in these three samples by this adsorbed antiserum, using the blotting technique after agarose electrophoresis (compare Fig. 1b).

*IV. Identification of the purified basic protein and other immunologically related proteins in seminal fluids*

Using immunodetection after SDS-PAGE, only trace amounts of the 12.8 kDa basic protein could be found in seminal vesicle secretion (Fig. 3c) and immunodetection after electrophoresis failed to detect it at all (Fig. 1b). In reduced seminal vesicle secretion, three dominant proteins, with apparent molecular masses of 52 kDa, 71 kDa, and 76 kDa (Fig. 3b,c), were identified after SDS-PAGE using the antiserum raised against the purified basic protein. In prostatic secretion (Fig. 3a,b), trace amounts of a 12.8 kDa protein were identified by this antiserum after SDS-PAGE (Fig. 3c), but no other proteins were detected. After incubating seminal vesicle secretion with a smaller volume of prostatic secretion (at 37°C for 30 min), immuno-

TABLE I. Amino acid composition of the purified basic protein

| Amino acid | nmol/ sample | Residue/ mol | Nearest integer |
|---|---|---|---|
| Aspartic acid | 74.5 | 13.0 | 13 |
| Threonine | 15.3 | 2.7 | 3 |
| Serine | 77.4 | 13.6 | 14 |
| Glutamic acid | 84.0 | 14.7 | 15 |
| Proline | 19.3 | 3.4 | 3 |
| Glycine | 100.9 | 17.7 | 18 |
| Alanine | 14.5 | 2.5 | 3 |
| Valine | 29.0 | 5.1 | 5 |
| Isoleucine | 22.8 | 4.0 | 4 |
| Leucine | 4.5 | 0.8 | 1 |
| Tyrosine | 10.1 | 1.8 | 2 |
| Phenylalanine | 12.6 | 2.2 | 2 |
| Lysine | 70.5 | 12.3 | 12 |
| Histidine | 85.8 | 15.0 | 15 |
| Arginine | 35.4 | 6.2 | 6 |
| Tryptophan | — | 0.8 | 1 |
| Total | 656.6 | | 117 |

Two samples of carboxymethylated protein (250 μg each) were dissolved in 100 μl 6 mol/l HCl and hydrolysed at 110°C for 24 or 72 h. The presented values for serine and threonine are the estimated values for O h hydrolysis. The tryptophan content was estimated from the UV absorbance spectrum of the protein in 6 mol/l guanidin HCl. The minimum number of residues in the protein was calculated from the apparent molecular mass of 12.8 kDa estimated on SDS-PAGE of the purified protein.

detection after SDS-PAGE (Fig. 3b,c) showed that the 52, 71, and 76 kDa proteins had disappeared, while the 12.8 kDa protein appeared together with several other related proteins having apparent molecular masses below 18 kDa. These proteins were also immunologically detected after SDS-PAGE of reduced seminal plasma (Fig. 3b,c). After agarose electrophoresis (Fig. 3a), prostatic fluid-incubated seminal vesicle secretion also showed a close similarity to seminal plasma with appearance of the predominant basic protein. After agarose electrophoresis of seminal plasma, four basic proteins were found to be immunologically related to the purified 12.8 kDa basic protein (Fig. 1a,b).

### *V. Amino acid composition of the purified basic protein*

The amino-acid composition presented in Table I is the corrected result of one 24-h and one 72-h hydrolysis of the 12.8 kDa basic protein. The values for serine and threonine are the calculated result for 0-h hydrolysis. Two more 24-h hydrolyses of separate preparations of the purified protein gave results closely agreeing with those presented. A tyrosine/tryptophan ratio of 2.30 was calculated from the UV absorbance spectrum of the protein. The protein contained no cysteine or methionine, nor, according to amino acid analysis, any glucosamine or galactosamine.

### *VI. $NH_2$-terminal sequence of the purified basic protein*

The results from Edman degradations of the $NH_2$-terminal of the 12.8 kDa basic protein are presented in Table II.

A single sequence was obtained throughout and, according to the Dayhoff data-base, it is not homologous to any published protein sequence. As presented here, the sequence renders improbable any common ancestry of the 12.8 kDa basic protein.

## DISCUSSION

The method presented here for purifying the predominant basic seminal plasma protein gave a single, homogenous product when examined by SDS-PAGE, agarose electrophoresis, and FPLC reversed-phase chromatography. Owing to its

TABLE II. Results of Edman degradations of the $NH_2$-terminal portion of the most basic, major seminal plasma protein

| 1 | 5 | 10 | 15 | 20 | 25 |
|---|---|---|---|---|---|
| His-Asn-Lys-Gln-Glu-Gly-Arg-Asp-His-Asp-Lys-Ser-Lys-Gly-His-Phe-His-Arg-Val-Val-Ile-His-His-Lys-Gly- | | | | | |

| 30 |
|---|
| Gly-Lys-Ala-His-Arg-Gly- |

lability, recovery of this protein from different chromatographic steps is dependent on repeated addition of serine protease inhibitors. Addition of benzamidine to buffers is also recommended, as the protein was labile even after partial purification.

The major part of the proteins in seminal plasma passed through the heparin-Sepharose column at low ionic strength, and we consider this to be an effective first step in purification. The basic protein was found to have an affinity to heparin slightly lower than that of antithrombin III [15]. In the subsequent gel filtration, polyacrylamide was found to be superior to agarose as the gel matrix, which might be explained by protein interaction with remaining sulphate groups in the agarose. It is possible to use HPLC gel filtration as a second step, but the low capacity of these columns is a distinct disadvantage. The final FPLC reversed-phase chromatography produced a homogenous protein that did not contain any detectable impurities, thus rendering it suitable for further characterization.

The coincidence between the results of HPLC gel filtration (unreduced protein), and those of SDS-PAGE (reduced and unreduced protein), suggests that the protein is a single polypeptide chain, a conclusion consistent with its lack of cysteine residues. The protein contains neither methionine nor carbohydrate; a basic antimicrobial protein, purified from bull semen, has been shown to be devoid of both cysteine and methionine [23], though it has a lower molecular mass (6.3 kDa), contains fewer histidine residues and a higher amount of leucine [23] than the human basic 12.8 kDa protein. The complete amino acid sequence of a rat 9.7 kDa basic seminal vesicle protein recently presented [20], is quite unlike the $NH_2$-terminal portion of the human 12.8 kDa protein; nor is this rat protein involved in semen coagulation [8]. In the rat, the clottable seminal vesicle protein [18] is a 37.9 kDa protein [7]; in the guinea pig, it is a 17.9 kDa protein [19] and, like the rat protein, it is devoid of cysteine, has a low tryptophan content, and is rich in glutamic and lysine residues [7, 19]. These characteristics are common to the human 12.8 kDa basic semen protein which, however, differs in its very high histidine content.

The 12.8 kDa basic human protein is unique to semen among all extracellular fluids examined, and it also has a unique $NH_2$-terminal amino acid sequence. The protein is very labile in seminal plasma, and is neither present in the supernatant of coagulated semen directly after liquefaction [12, 13], nor in seminal vesicle or prostatic secretion in significant concentration. An immunologically related seminal vesicle protein which, when reduced, was identified after SDS-PAGE, as three high molecular weight proteins, was the predominant protein in this secretion. One or more prostatic enzymes were able to degrade this predominant seminal vesicle protein to several basic, low molecular weight proteins which are quantitatively predominant in seminal plasma. The purified 12.8 kDa protein is the most basic of these and the predominant basic protein in seminal plasma. In an accompanying paper [12] we present evidence for the release of these proteins during the liquefaction of coagulated human semen.

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# Amino acid sequence of the predominant basic protein in human seminal plasma

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The predominant basic protein in liquefied human seminal plasma is the major degradation product of the gel-forming protein secreted by the seminal vesicles. The amino acid sequence of this basic protein is presented. The basic protein contains 52 amino acid residues. It is devoid of cysteine, methionine, tryptophan, and leucine, but contains seven histidine residues located in the $NH_2$-terminal half of the molecule. The calculated $M_r$ of 5753 is in close agreement with that obtained from gel filtration in guanidine-HCl on Sephacryl S-200 ($M_r = 6000$).

*Amino acid sequence* *Inhibin* *Seminal plasma* *Seminal vesicle*

## 1. INTRODUCTION

The coagulate structure of freshly ejaculated semen is liquefied within 15 min by prostatic enzyme(s) [1]. It has been proposed that the protein predominant in seminal vesicle secretion is the structural protein of the coagulate [2], and that during liquefaction this protein is cleaved into a number of basic proteins, the major one of which has recently been purified [3]. As estimated using SDS-polyacrylamide gel electrophoresis (SDS-PAGE), the protein's $M_r$ is about 12800. Its $NH_2$-terminal sequence, comprising 31 identified amino acid residues, is unique according to the Dayhoff data base [3]. Seidah et al. [4] recently presented an identical $NH_2$-terminal sequence of a basic peptide, also purified from human seminal plasma and consisting of about 35 amino acid residues, and which was found to inhibit secretion of rat pituitary follicle-stimulating hormone. A synthetic replica of this $NH_2$-terminal sequence has been shown to have a similar inhibitory capacity [5]. Here, we report the complete sequence of the predominant basic protein in human seminal plasma.

## 2. MATERIALS AND METHODS

### 2.1. *Chemicals, separation materials, and enzymes*

Sephacryl® S-200 and FPLC® system equipped with an HR 5/5 PepRPC($C_2/C_{18}$) column were from Pharmacia (Uppsala); Ultropac® TSK G 2000 SW column from LKB (Bromma, Sweden); diisopropylfluorophosphate (DFP) from Sigma (St Louis, MO); trypsin treated with L-(tosylamide-2-phenyl)ethyl chloromethyl ketone (TPCK) from Worthington (Freehold, NJ). Carboxypeptidase Y was a gift to Professor J. Stenflo from Professor M. Ottesen, Carlsberg Laboratories (Copenhagen, Denmark).

### 2.2. *Sample, material, purifications, preparation of fragments, and COOH-terminal analysis*

The predominant basic protein was purified from pooled human seminal plasma as in [3]. Lysine-blocked protein was prepared by treatment with citraconic anhydride (30 mol/mol purified protein) according to Atassi and Habeeb [6]. Tryptic digestion of intact or lysine-blocked protein (3 mg/ml) in 0.1 M $NH_4HCO_3$ was carried out at 37°C for 3 h, at an enzyme:substrate ratio of

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1:20. TPCK-trypsin was inactivated by DFP (20 mM). The digestion products of the lysine-blocked protein were kept at pH 4 for 3 h at 37°C to deblock the amino groups before separation. Fragments obtained were purified by reversed-phase chromatography (RPC) as described in the legend to fig.2. Intact protein or isolated fragments were digested with carboxypeptidase Y [7] as described in the legend to fig.3.

### 2.3. $M_r$ *estimation of purified predominant basic protein*

Reduced and carboxymethylated proteins were gel filtered on a Sephacryl S-200 column (1749 × 16 mm) equilibrated in 0.25 M sodium phosphate with 6 M guanidine-HCl, pH 7.4 [8]. HPLC gel filtration in 0.1 M sodium phosphate with 6 M guanidine-HCl, pH 6.9, was performed in a TSK G 2000 SW column at a flow rate of 0.5 ml/min.

### 2.4. *Amino acid analysis*

Amino acid analysis was performed on a Beckman 119 CL analyser after hydrolysis of fragments in 6 M HCl at 110°C for 24 h [9]. Hydrolysis in 4 M methanesulphonic acid for tryptophan analysis was performed according to Inglis [10].

### 2.5. *Sequence determination*

Sequence determination by automatic Edman degradations was performed in a Beckman 890 C sequencer. The Beckman no.127974 Quadrol program was used and 2 mg polybrene was added to the spinning cup. PTH amino acids were identified by HPLC [11].

## 3. RESULTS

### 3.1. *$NH_2$-terminal sequence degradation of intact protein*

Automatic Edman degradations of the intact protein (20 and 90 nmol) allowed identification of 31 $NH_2$-terminal residues and separate residues to position 45 (fig.1).

### 3.2. *Tryptic fragments*

Tryptic fragments were purified by RPC (fig.2a). Fragments TR0–TR4 were hydrolysed and amino acid analysis showed an equal molar yield for all fragments (65% of theoretical yield).

Fig.1. Amino acid sequence of the predominant basic protein in human seminal plasma. The sequence is presented in the standard IUPAC, one-letter code for amino acid residues. The numbering of tryptic fragments (TR1–TR4) and the numbering of fragments of lysine-blocked protein (ARG1–ARG4) are explained in fig.2. (●) Residues identified by sequenator analysis; (○) residues not identified by sequenator analysis; (⟷) residues identified by carboxypeptidase Y digestion.

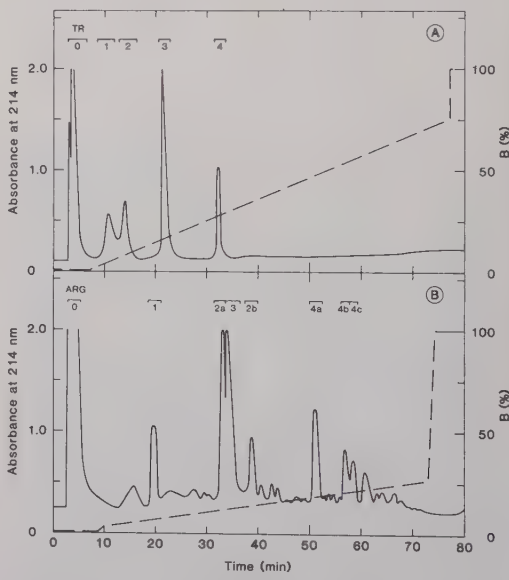


Fig.2. Reversed-phase chromatography on an HR 5/5 Pep RPC ($C_2/C_{18}$) column at a flow rate of 0.5 ml/min. Elution was performed with a 80 min gradient from 0 to 25% B, or from 0 to 75% B; solvent A, 0.1% (v/v) trifluoroacetic acid in water; solvent B, 80% (v/v) acetonitrile–0.1% (v/v) trifluoroacetic acid; (A) TR fragments obtained from tryptic digestion of intact protein; (B) ARG fragments obtained from tryptic digestion of lysine-blocked protein.

The molar ratios of the amino acids in TR0 corresponded to those of the residues constituting position 1–11 plus 25–30 (fig.1). Sequenator analysis of TR1, TR2, and TR4 allowed identification of all residues. Ten $NH_2$-terminal residues in TR3 were identified by sequenator analysis.

### 3.3. *Fragments of lysine-blocked protein*

Fragments obtained by tryptic digestion of citraconylated protein were purified by RPC (fig.2b). ARG0 contained no amino acids. ARG1–ARG4 fragments were obtained in equal molar yields (50% of theoretic yield). Amino acid analysis of ARG1, ARG2a, ARG2b, and ARG3 allowed their identification (fig.1). Sequenator analysis of ARG4a (15 nmol) allowed identification of all residues except those at positions 13 and 20. The coinciding results from amino acid analysis of ARG4a, ARG4b, and ARG4c were consistent with the presence of proline and serine in these positions (fig.1). Carboxypeptidase Y digestion liberated tyrosine as the COOH-terminal residue from ARG4a, 4b, and 4c.

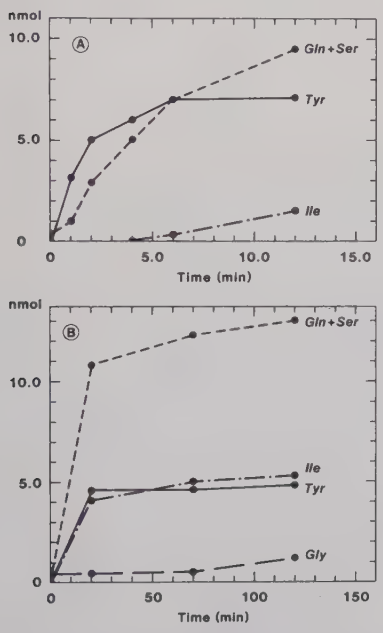


Fig.3. Liberated amino acids after digestion of intact protein with carboxypeptidase Y at an enzyme : substrate ratio of 1 : 30 or 2 : 30. Digestion was performed in 0.1 M pyridine-acetate, pH 5.5, at 37°C. Reaction was stopped by adding equal volume of 6% (w/v) sulphosalicylic acid to the vial which was then immediately placed in boiling water and then centrifuged. The separated supernatant was analysed on a Beckman 6300 amino acid analyser; (A) 8 nmol of intact protein digested for 0–12 min; (B) 6 nmol of protein digested for 0–120 min.

### 3.4. *COOH-terminal analysis of intact protein*

COOH-terminal residues were identified by following the release of amino acids during carboxypeptidase Y digestion of intact protein (fig.3a and b).

### 3.5. $M_r$ *estimation of purified predominant basic protein*

Gel filtration in 6 M guanidine-HCl on Sephacryl S-200 gave the purified protein an $M_r$ of 6000 (not shown). The protein eluted, however, in two distinct peaks from a TSKG 2000 SW column equilibrated in 6 M guanidine-HCl (not shown).

Table 1

Amino acid composition of the purified predominant basic protein

| Amino acid | nmol/ sample | Residue/ mol | Sequence |
|---|---|---|---|
| Aspartic acid | 229.9 | 6.3 | 6 |
| Threonine | 38.6 | 1.1 | 1 |
| Serine | 198.1 | 5.4 | 6 |
| Glutamic acid | 228.6 | 6.2 | 6 |
| Proline | 76.7 | 2.1 | 2 |
| Glycine | 298.3 | 8.1 | 8 |
| Alanine | 39.9 | 1.1 | 1 |
| Valine | 48.4 | 1.3 | 2 |
| Isoleucine | 67.6 | 1.8 | 2 |
| Tyrosine | 34.2 | 0.9 | 1 |
| Phenylalanine | 41.2 | 1.1 | 1 |
| Lysine | 223.5 | 6.1 | 6 |
| Histidine | 269.2 | 7.3 | 7 |
| Arginine | 116.5 | 3.2 | 3 |
| Total | 1910.7 | | 52 |

430 $\mu$g of intact protein was hydrolysed in 4 M methanesulphonic acid at 110°C for 24 h. Hydrolysate was analysed on the Beckman 6300 analyser. No tryptophan was found in the sample

The first peak eluted with the same retention as carboxymethylated cytochrome *c* ($M_r$ = 12800), and the second peak eluted with the same retention as carboxymethylated aprotinin ($M_r$ = 6400).

### 3.6. *Complete amino acid sequence of the predominant basic protein in seminal plasma*

The amino acid residues identified by $NH_2$-terminal analysis in intact protein provide considerable overlap into ARG4. The position of TR4 as the COOH-terminal fragment is well substantiated. The heterogeneity of ARG4 fragments might be explained by deamidation of glutamine residues. All peptides isolated are consistent with the sequence deduced (fig.1). Amino acid analysis of the intact protein after hydrolysis in 4 M methanesulphonic acid (table 1) agrees closely with the sequence presented. The protein contains 52 amino acid residues, from which its $M_r$ is calculated to be 5753.

## 4. DISCUSSION

The calculated $M_r$ (5753) of the predominant basic protein is in close agreement with that obtained by gel filtration on Sephacryl S-200 in 6 M guanidine-HCl. Analysis with SDS-PAGE has previously given the protein an $M_r$ of 12800, and HPLC gel filtration in 0.1 M ammonium acetate at pH 5.5 an $M_r$ of 15000 [3]. Upon HPLC gel filtration in 6 M guanidine-HCl, the purified protein eluted in two peaks, the first of which eluted at a position corresponding to twice the $M_r$ of the protein sequence. These data suggest the existence of a dimeric form of the purified protein which is remarkably stable under denaturing conditions.

According to the Protein Sequence Database of the Protein Identification Resource (Washington, DC), the sequenced protein has a 47% identity with a histidine-rich portion of the bovine type I precursor of high-$M_r$ kininogen in an overlap of 17 amino acid residues. In an overlap of 42 residues, the predominant basic protein has a 21% identity with the bovine type II precursor of high-$M_r$ kininogen. Furthermore the protein has a 30% identity with human histone 2A in an overlap of 30 residues, and a 34% identity with DNA-dependent RNA polymerase (EC 2.7.7.6) in an overlap of 29 residues. The predominant basic protein in seminal plasma is a major degradation product of a high-$M_r$ precursor secreted by the seminal vesicles [3], and is very labile in seminal plasma [12]. Seidah et al. [4] have purified and partly characterised an inhibin-like basic peptide, whose $NH_2$-terminal sequence is identical with that presented here, and which is considered to be a degradation product of the predominant basic protein in seminal plasma.

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# Liquefaction of coagulated human semen

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Liquefaction of coagulated human semen was inhibited by *o*-phenanthroline; subsequent addition of $Zn^{2+}$ reversed this inhibition, but not if the coagulum was repeatedly washed before $Zn^{2+}$ was added. No liquefaction of the coagulum occurred when $Fe^{2+}$ was added (in a 1:3 molar ratio to *o*-phenanthroline), and the gel repeatedly washed. This *o*-phenanthroline-depleted coagulum was liquefied by resuspended pellet from ultracentrifuged pooled seminal plasma.

In denatured and reduced semen coagulate, we identified the 52 kDa, 71 kDa, and 76 kDa protein bands of the predominant seminal vesicle protein. The protein was not present in the supernatant after centrifugation of coagulated semen, and it was degraded to several minor basic proteins when semen liquefied. These findings imply that the predominant seminal vesicle protein functions as the structural protein of coagulated semen. In much the same way as in ejaculated semen, the 52 kDa, 71 kDa, and 76 kDa protein bands in seminal vesicle secretion collected post-mortem were digested to minor basic proteins after incubating the secretion with resuspended pellet from ultracentrifuged seminal plasma. This pellet contained the membrane-bound succinyl(alanine)$_3$-*p*-nitroanilide hydrolysing peptidase of prostatic origin which, like the liquefaction process, was active in the presence of EGTA, inhibited by non-chelated $Zn^{2+}$, and inactivated by *o*-phenanthroline—an inactivation that was reversed by $Zn^{2+}$.

*Key-words:* membrane proteins; peptidohydrolase; peptide fragments; protease inhibitors; prostate; semen; seminal vesicles

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The first portion of ejaculated semen, in which components secreted by the prostate gland predominate, also contains most spermatocytes. The last portion is largely composed of a secretion from the seminal vesicles [7]. Upon ejaculation, semen immediately turns into a loose coagulum with entrapped spermatozoa. Components from the seminal vesicles are considered to generate the coagulum matrix [8]. Congenital aplasia of the seminal vesicles is connected with inability to form a seminal coagulum on ejaculation [1]. The merging of seminal vesicle secretions and those of other accessory sex glands or spermatozoa (or both), is held to be quite immaterial in gel formation [14]. The structural protein(s) of the human coagulum has yet to be characterized, but the mechanism of semen coagulation has been clarified in both the guinea pig [15] and in the rat [16]; transglutaminases, secreted by the anterior prostate (the coagulating gland), have been shown to form intermolecular γ-glutamyl-ε-lysine cross-links between basic

proteins that are secreted by the seminal vesicles. Neither transglutaminase activity, nor factor XIII has been reported to be present in human semen [13].

One or more components present in the prostatic secretion are thought to account for the liquefaction of coagulated semen, a lysis normally complete within twenty minutes of ejaculation [12]. A neutral protease known as seminin [11], and a metallo-dependent collagenase-like peptidase [6] have been proposed as agents in the liquefaction process [6, 11, 14].

We recently reported that the metal chelator, *o*-phenanthroline, completely inhibited liquefaction of coagulated semen, whereas serine or thiol inhibitors did not [5]. A recently described membrane-bound peptidase of prostatic origin [3] is inactivated by *o*-phenanthroline but not by $Na_2EDTA$. This peptidase is characterized by its succinyl(alanine)$_3$-*p*-nitroanilide hydrolysing activity [3].

The aim of the present investigation was to study the metal ion dependence of the liquefaction process, the proteins in the coagulum and the cleavage products formed during lysis.

## MATERIAL AND METHODS

*Chemicals.* *O*-phenanthroline-HCl was obtained from E. Merck (Darmstadt, FGR), diisopropylfluorophosphate (DFP) from Sigma Chemical Co. (St Louis, Mo., USA), and succinyl (alanine)$_3$-*p*-nitroanilide (Suc(Ala)$_3$pNA) from Protein Research Foundation (Minoh, Shi, Osaka, Japan). The molecular weight determination kit was obtained from Pharmacia Fine Chemicals (Uppsala, Sweden). All other chemicals used were of reagent grade.

*Sample material.* Human semen, from patients undergoing investigation for involuntary infertility or from voluntary donors, was obtained from the fertility laboratories in Malmö, Sweden, all specimens having been collected by masturbation. The samples were allowed to liquefy at room temperature. Seminal plasma was then obtained as the separated supernatant after centrifugation of semen, within 5 min of liquefaction at 800 *g* for 15 min, and then frozen at −20°C if not used immediately. Coagulated semen, having an estimated normal liquefaction rate at room temperature, was collected in vessels that were placed in ice-cold $H_2O$ immediately after ejaculation. Each coagulated sample was cyclomixed and then, in certain cases after adding protease inhibitor, either used immediately or frozen at −70°C until required. Seminal vesicle secretion, collected post-mortem from three men, 39, 65, and 66 years old, were sampled at autopsy within 12 h of death and immediately frozen at 70° C until used.

*Estimation of liquefaction of coagulated semen.* All incubation experiments were performed at 37°C, if not otherwise stated. The samples were slowly rocked, and liquefaction was estimated to have occurred when gel-clumps were no longer observed, and the fluid semen could be drawn into a Pasteur pipette.

*Ultracentrifugation.* Pooled seminal plasma (10 ml) was centrifuged at 100,000 *g* for 1 h at 4°C. The supernatant was discarded and the remaining, separated pellet (about 0.2 ml) was resuspended in 0.5 ml of 50 mmol/l Tris, pH 8.0, with 0.15 mol/l NaCl. This pellet suspension was then used for incubation experiments.

*Suc(Ala)$_3$pNA hydrolysing activity.* This activity measurement was monitored at 405 nm at room temperature as previously described [3].

*Purification of the predominant basic seminal plasma protein.* The purification of this 12.8 kDa protein, and the raising of a specific rabbit antiserum (anti-12.8 kDa bsp) against it, are described in the accompanying paper [4].

*Electrophoretic techniques.* Sodium dodecylsulphate-polyacrylamide gel electrophoresis (SDS-PAGE) of samples reduced with dithiothreitol and denatured in heated SDS, immunodetection with Western blotting after SDS-PAGE, and agarose electrophoresis, were performed as described in the accompanying paper [4].

## RESULTS

### *A. Liquefaction of coagulated semen*

*I. Inhibition and reactivation of liquefaction.* Addition of *o*-phenanthroline to coagulated semen, to a final concentration of 50 mmol/l, completely inhibited liquefaction of the gel. This *o*-phenanthroline inhibition could be reversed by adding 3–6 mmol/l of $Zn^{2+}$ to the samples. With the addition of the $Zn^{2+}$, the liquefaction rate was reduced, and more than 80% of the coagulum volume lysed within 80–110 min ($n = 4$). The liquefaction time of the gels was 5–16

min ($n = 4$) in absence of inhibitor. Coagulated semen that contained neutrally buffered EGTA, at a final concentration of 50 mmol/l, liquefied completely after 4–9 min ($n = 4$). When about 1 ml of coagulated semen, to which *o*-phenanthroline had been added, was washed repeatedly (5 × 10 ml) with either Tris buffer (50 mmol/l, pH 8.0, with 0.15 mol/l NaCl) or distilled $H_2O$, before adding $Zn^{2+}$ (3–6 mmol/l), the gels did not liquefy at all ($n = 4$).

*II. Liquefaction of washed coagulated semen.* Neutralization and reduction of the *o*-phenanthroline content (50 mmol/l) in coagulated semen was accomplished by adding $Fe^{2+}$ (16 mmol/l), followed by repetitive washing of the gel with distilled $H_2O$ for 24 h. No liquefaction of the coagulated semen occurred, and the *o*-phenanthroline elimination was estimated by the formation of the coloured [$Fe^{2+}$ (*o*-phenanthroline)$_3$] -complex. Gels thus depleted of *o*-phenanthroline liquefied after the addition of resuspended pellet from ultracentrifuged pooled seminal plasma.

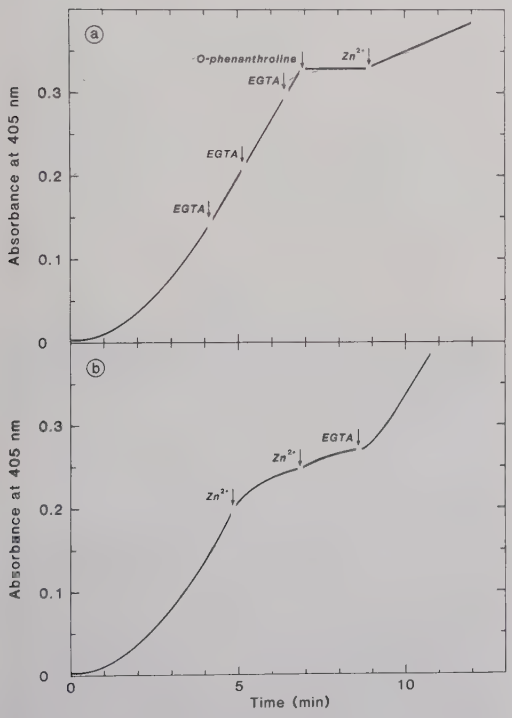


FIG. 1. Suc(Ala)$_3$pNA hydrolysing activity measured in 1 ml of substrate solution at 405 nm of 15 µl of resuspended pellet from ultracentrifuged pooled seminal plasma (a) after addition of 5 µmol of $Na_2$-EGTA (pH 8), 15 µmol of *o*-phenanthroline, and 2 µmol of $Zn^{2+}$; and (b) after addition of 5 µmol of $Zn^{2+}$, and 20 µmol of $Na_2$-EGTA (pH 8).

### *B. Metal ion dependence of Suc(Ala)$_3$pNA hydrolysing peptidase*

A resuspended pellet from ultracentrifuged pooled seminal plasma was monitored for Suc(Ala)$_3$pNA hydrolysing activity (Fig. 1a) after the addition of $Na_2$-EGTA (pH 8), *o*-phenanthroline, and $Zn^{2+}$. The membrane-bound Suc(Ala)$_3$pNA peptidase was not inhibited by increasing the concentrations of EGTA, but it was totally inactivated by *o*-phenanthroline. The addition of $Zn^{2+}$ to the solution reactivated the peptidase, but the initial rate of Suc(Ala)$_3$pNA hydrolysing activity was not regained in the presence of *o*-phenanthroline.

Addition of $Zn^{2+}$ to peptidase-active pellet suspension (Fig. 1b) partially inhibited the Suc(Ala)$_3$pNA hydrolysing peptidase. The inhibition by excess $Zn^{2+}$ could be completely reversed by the addition of $Na_2$-EGTA (pH 8).

### *C. Identification of a major seminal vesicle protein in coagulated semen and its degradation during liquefaction*

The proteins in seminal vesicle secretion and in coagulated and liquefied semen were studied by SDS-PAGE (Fig. 2a), and with Western blotting immunodetection after SDS-PAGE using the specific anti-12.8 kDa bsp rabbit serum (Fig. 2b). When denatured and reduced, seminal vesicle secretion, collected post-mortem, contained three immunologically related proteins—52 kDa, 71 kDa, and 76 kDa—as did *o*-phenanthroline-treated coagulated semen (0.5 ml) that had been repeatedly washed with (5 × 10 ml) distilled $H_2O$. Some immunologically related proteins with intermediate molecular masses were present in coagulated semen, whereas the seminal vesicle secretion gave no distinct protein bands in this region. Separated supernatants, obtained after centrifugation of coagulated semen (at 3000 *g* for 2 min), contained no detectable amounts of the 52 kDa, 71 kDa, and 76 kDa seminal vesicle proteins, but did contain minute amounts of the immunologically related predominant 12.8 kDa basic seminal plasma protein. The 52 kDa, 71 kDa, and 76 kDa seminal vesicle protein bands

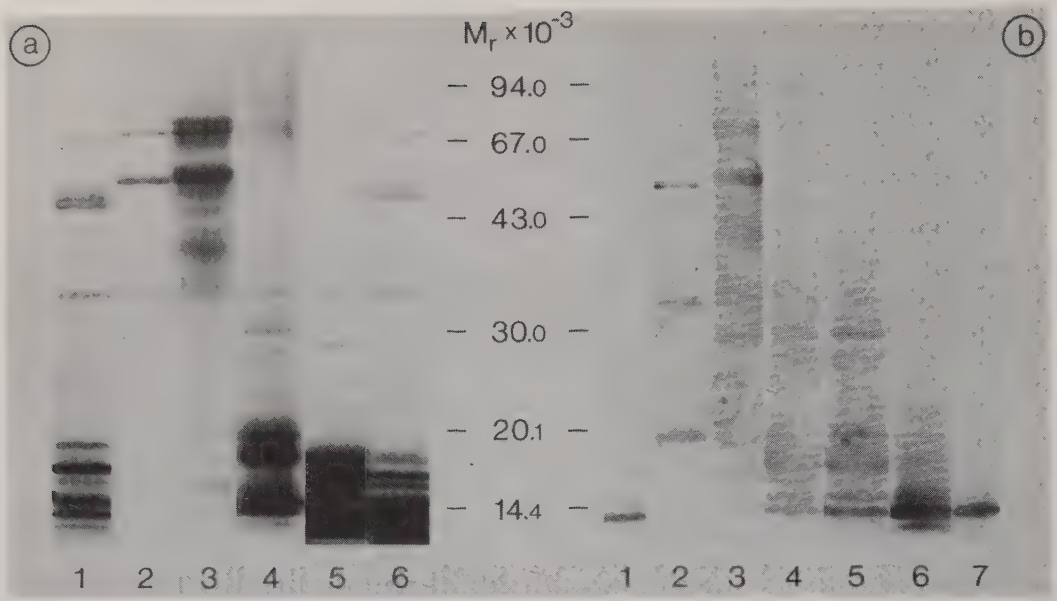


FIG 2a. SDS-PAGE in a 10–15% (w/v) gel gradient run at 20 mA for 16 h. Reduced samples with a volume of 80 μl were applied to each lane. All samples were estimated to have a protein content of 15–20 μl except in lane 2 where such an estimation was not done because of the viscosity of this sample. 1, Supernatant of centrifuged coagulated semen; 2, repeatedly washed *o*-phenanthroline-treated coagulated semen; 3, seminal vesicle secretion, collected post-mortem; 4, seminal vesicle secretion (20 μl) incubated (at 37°C for 30 min) with resuspended pellet (10 μl) of ultracentrifuged seminal plasma; 5, seminal plasma liquefied in the presence of 10 mmol/l DFP; 6, seminal plasma liquefied in the absence of any exogenous protease inhibitor. (b) Immunodetection with the anti-12.8 kDa bsp rabbit serum after SDS-PAGE. 1, Supernatant of centrifuged (3000 *g*) coagulated semen; 2, repeatedly washed *o*-phenanthroline-treated (50 mmol/l) coagulated semen; 3, seminal vesicle secretion, collected post-mortem; 4, seminal vesicle secretion (20 μl) incubated at 37°C for 30 min with resuspended pellet (10 μl) of ultracentrifuged seminal plasma; 5, seminal plasma liquefied in the presence of 10 mmol/l DFP; 6, seminal plasma liquefied in the absence of any exogenous protease inhibitor; 7, purified 12.8 kDa basic protein.

had disappeared both in seminal plasma liquefied in absence of any exogenous protease inhibitor, and in seminal plasma liquefied in the presence of 10 mmol/l DFP. *O*-phenanthroline-treated coagulated semen that had liquefied after the addition of $Zn^{2+}$ contained minute amounts of the 52 kDa, 71 kDa, and 76 kDa protein bands (not shown). In all these liquefied samples, immunologically related proteins appeared with molecular masses below 18 kDa, of which the 12.8 kDa basic protein is one. These minor basic proteins were less degraded (i.e. had higher molecular masses) in the samples that contained protease inhibitors than in that liquefied in the absence of any exogenous protease inhibitor.

Seminal vesicle secretion, collected post-mortem, that was incubated with the resuspended pellet of ultracentrifuged seminal plasma contained none of the 52 kDa, 71 kDa, and 76 kDa seminal vesicle proteins (Figs 2a and b). Instead the immunologically related proteins with molecular masses below 18 kDa appeared, making this pattern similar to that of liquefied seminal plasma.

## DISCUSSION

We have previously reported that several basic proteins are released during liquefaction of coagulated semen [5]. The predominant of these basic proteins has been purified and partly characterized, and the majority of the other basic seminal plasma proteins were shown to be immunologically related to this 12.8 kDa protein (see accompanying paper [4]). It was also found that these basic proteins are degradation products of the predominant seminal vesicle protein that, when reduced and denatured, produced the 52 kDa, 71 kDa, and 76 kDa

proteins [4]. As shown here, this seminal vesicle protein is present in its original high molecular weight form in coagulated semen, but missing in the supernatant after centrifugation of coagulated semen. Liquefaction of semen apparently implied degradation of the large vesicular protein into a series of minor proteins. After complete liquefaction of semen, these basic proteins constitute a major part of the seminal plasma proteins. From these observations we conclude that the predominating seminal vesicle protein functions as the structural protein of coagulated human semen, perhaps after some cross-linking. The close resemblance between the protein pattern of prostatic secretion [4] and the supernatant of centrifuged coagulated semen, and the resemblance of the semen coagulate with the seminal vesicle secretion, are consistent with the seminal vesicle secretion being expelled as a preformed clot. There are no previous reports on the nature of any structural proteins in coagulated human semen but in the rat [9] and in the guinea pig [10] the clottable proteins are basic seminal vesicle proteins that are rich in glutamic and lysine residues, but devoid of cysteine. These constituents are common to the 12.8 kDa protein in seminal plasma [4], which is the predominant degradation product of the major human seminal vesicle protein. The human 12.8 kDa basic protein has very high histidine content as compared to the rat and guinea pig proteins. Whether this difference is related to the lysis of the human coagulum, in contrast to the clot stability in the rat or guinea pig, remained to be ascertained. Transglutaminases polymerize the clottable proteins in the guinea pig [15] and in the rat [16]. No factor XIII has been detected immunochemically in human semen [13], but no thorough search has yet been made for other transglutaminases in human ejaculates collected under controlled conditions.

According to Koren & Lukač [2], liquefaction of semen occurs at a higher rate in the presence of EDTA, and our results suggest that EGTA has a similar effect. Koren & Lukač also showed that liquefaction was inhibited in the presence of $Zn^{2+}$ and suggested that an EDTA-sensitive collagenase-like peptidase [5] was mainly responsible for semen liquefaction, a suggestion that seems improbable in view of our results. Like the semen liquefaction process, our organelle-bound $Suc(Ala)_3pNA$ hydrolysing peptidase [3] is (a) not inhibited by EDTA or EGTA, (b) inactivated by *o*-phenanthroline, (c) reactivated by $Zn^{2+}$, and (d) inhibited in the presence of non-chelated $Zn^{2+}$. Seminal organelle suspensions with high peptidase activity were found to liquefy coagulated semen and degrade the predominant seminal vesicle protein to basic proteins with low molecular masses. These data indicate the participation of the $Suc(Ala)_3pNA$ hydrolysing peptidase in semen liquefaction, but the full determination of its exact role requires further characterization of the enzyme.

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# The predominant protein in human seminal coagulate

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The predominant protein in human seminal vesicle secretion constitutes the structural protein of coagulated semen. This high molecular weight protein (HMW-SV-protein) is stable in seminal vesicle secretion during in vitro storage at 37 °C for at least 20 h, but is rapidly cleaved on mixing with prostatic proteases. Seminal coagulate, washed free of souble components, is dissoluble by 2 to 3 mol/l of guanidine-HCl. Although dithiothreitol added to seminal coagulate does not liquify the clot, complexes between HMW-SV-proteins are broken up by reduction under denaturing conditions, which suggests that the non-covalent linkages of HMW-SV-proteins are essential in the clot. Prostatic proteases cleave the HMW-SV-protein during liquefaction of ejaculated semen to a series of labile proteins. These proteins are further cleaved to peptides of successively decreasing size after completed liquefaction. The cleavage of the HMW-SV-protein is the major cause of the fast shift of the electrophoretic pattern of seminal proteins if semen is stored without protease inhibitors.

*Key words*: inhibin; prostate; protease inhibitors; proteolytic enzymes; semen

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The seminal vesicle secretion contains the structural basis for the immediate coagulation of ejaculated human semen [1]. The predominant protein in seminal vesicle secretion is the structural protein of the seminal clot [2]. This high molecular weight protein, known as HMW-SV-protein, gives major protein bands of about 52, 71, and 76 kDa on sodium dodecylsulphate polyacrylamide gel electrophoresis (SDS-PAGE) in the presence of reducing agents. The clot normally liquefies within 20 min of ejaculation, when the HMW-SV-protein is cleaved to low molecular weight proteins that dissolve in the seminal fluid [2, 3]. One major, relatively stable, cleavage product is a very basic protein with an apparent mass of 12.8 kDa according to SDS-PAGE [4], and a mass of 5.8 kDa according to its complete amino acid sequence [5]. The $NH_2$-terminal portion of the basic protein inhibits the secretion of rat pituitary, follicle-stimulating hormone [6]. In semen, proteolytic degradation, particularly of the basic proteins, proceeds after complete liquefaction of the clot. This is reflected by rapid changes of the electrophoretic protein pattern, and by a rapid increase of the non-protein nitrogen content of semen (reviewed by Mann [7]). Inhibition of this proteolysis might be achieved by the addition to semen of either thiol reagents, serine protease inhibitors, or o-phenanthroline [8, 9]. The aim of the present investigation was

to study the HMW-SV-protein in human seminal clots and to identify its cleavage products during liquefaction.

## MATERIALS AND METHODS

*Chemicals.* Ortho-phenanthroline-HCl, dithiothreitol (DTT), and iodoacetic acid were obtained from E. Merck (Darmstadt, FRG); molecular weight markers for polyacrylamide gel electrophoresis from Pharmacia Fine Chemicals (Uppsala, Sweden); acrylamide (electrophoresis grade), and N'N-methylene-bisacrylamide from British Drug Houses (Poole, UK); 3-amino-9-ethylcarbazole and di-isopropylfluorophosphate (DFP) from Sigma Chemical Co. (St. Louis, Mo., USA), and Isogel® agarose from FMC Corp., Marine Colloids Division (Rockland, Me., USA). Peroxidase coupled goat anti-rabbit IgG was available at the laboratory.

*Material.* Human semen, from voluntary donors, was frozen directly after ejaculation, some samples with protease inhibitors and some without, as earlier described [9]. Secretion, aspirated from the seminal vesicle ducts of a male patient during an operation for carcinoma in the urinary bladder, was frozen directly in liquid nitrogen and stored at −70 °C until required. Seminal vesicle secretion was collected post-mortem, and pure prostatic secretion as described previously [2].

*Preparation of clotted semen.* A pool of coagulated semen (about 8 ml) was prepared from samples to which o-phenanthroline-HCl (50 mmol/l) had been added before they were frozen. The clot was then washed free of soluble components by suspension in 0.15 mol/l NaCl (5 × 10 ml). The sample was centrifuged at 800 × g for 5 min, the supernatant being discarded after each washing cycle.

*Electrophoretic techniques.* Agarose gel electrophoresis was performed according to standard procedures [10]. The SDS-PAGE procedure, in 9–17% (w/v) polyacrylamide gel gradients, was performed according to Blobel & Dobberstein [11]. Immunoblotting after SDS-PAGE was carried out with the Western blot technique described by Burnette [12], while immunoblotting after agarose gel electrophoresis was performed essentially according to Glynn et al. [13]. A rabbit antiserum that is specific against the predominant basic seminal plasma protein was used as first antibody [4]. The immune complexes were detected with peroxidase-coupled goat anti-rabbit IgG. Visualization was made possible with 3-amino-

TABLE I. Amino acid composition of clotted semen, washed free of soluble components and then carboxymethylated. The cot was hydrolysed in 6 mol/l HCl at 110 °C for 24 h. The amino acid composition of the predominant basic seminal plasma protein [5] is given for comparison

| Amino acid | Seminal clot (nmol/sample) | Basic protein (residue/mol protein) |
|---|---|---|
| Carboxymethylcysteine | 0.8 | 0 |
| Aspartic acid | 6.7 | 6 |
| Threonine | 3.8 | 1 |
| Serine | 7.2 | 6 |
| Glutamic acid | 13.4 | 6 |
| Proline | 2.1 | 2 |
| Glycine | 6.1 | 8 |
| Alanine | 2.5 | 1 |
| Valine | 3.1 | 2 |
| Methionine | 0.3 | 0 |
| Isoleucine | 2.7 | 2 |
| Leucine | 4.1 | 0 |
| Tyrosine | 2.1 | 1 |
| Phenylalanine | 1.4 | 1 |
| Lysine | 6.1 | 6 |
| Histidine | 4.4 | 7 |
| Arginine | 3.2 | 3 |
| Tryptophan | Not determined | 0 |
| | 70 | 52 |

9-ethylcarbazole according to Graham et al. [14].

*Amino acid analysis:* Coagulated semen, washed free of soluble components, was reduced for 1 h with 25 mmol/l DTT in 0.5 mol/l TRIS-HCl with 6 mol/l guanidine-HCl, pH 8.0. The sample was then carboxymethylated with 50 mmol/l iodoacetic acid in 0.5 mol/l TRIS-HCl, pH 8.0, for 2 h in darkness. Portions (containing about 50 μg each) of the carboxymethylated clot were hydrolysed in 6 mol/l HCl in evacuated sealed tubes at 110 °C for 24 h. The dried hydrolysates were analysed in a Beckman 6300 amino acid analyser.

*Transglutaminase activity:* Activity staining for transglutaminase (EC 2.3.2.13) after agarose gel electrophoresis was performed according to Stenberg & Stenflo [15] with and without thrombin included in the activity staining solution. Using this method, a transglutaminase concentration above 5 nmol/l in seminal fluid could be detected.

## RESULTS

### *1. Effects on the seminal clot by reduction and denaturation*

Washed seminal clot, divided in portions of about 1 ml each, was suspended in (i) 1.0 ml of 0.5 mol/l TRIS-HCl, pH 8.0; (ii) 1.0 ml of 75 mmol/l DTT in 0.5 mol/l TRIS-HCl, pH 8.0; (iii) 1.0 ml of 6 mol/l guanidine-HCl in 0.5 mol/l TRIS-HCl, pH 8.0. The clot dissolved immediately in the buffer containing guanidine, but remained unaltered for several hours at 37 °C in the other buffers. A final concentration of 2 to 3 mol/l of guanidine-HCl was found to liquefy the clot completely.

### *2. Amino acid analysis of clotted semen*

The amino acid composition of clotted semen, washed free of soluble components, had distinct similarities to the amino acid composition of the 5.8 kDa basic protein (a major intermediate cleavage product of the HMW-SV-protein). The clot contained very little cysteine and methionine, but in comparison with the 5.8 kDa basic protein, not only was there a much higher content of glutamic acid in the clot, but it also contained leucine (Table I).

### *3. SDS-PAGE of HMW-SV-protein*

Analysis of seminal vesicle secretion or washed seminal clot by SDS-PAGE (Fig. 1)

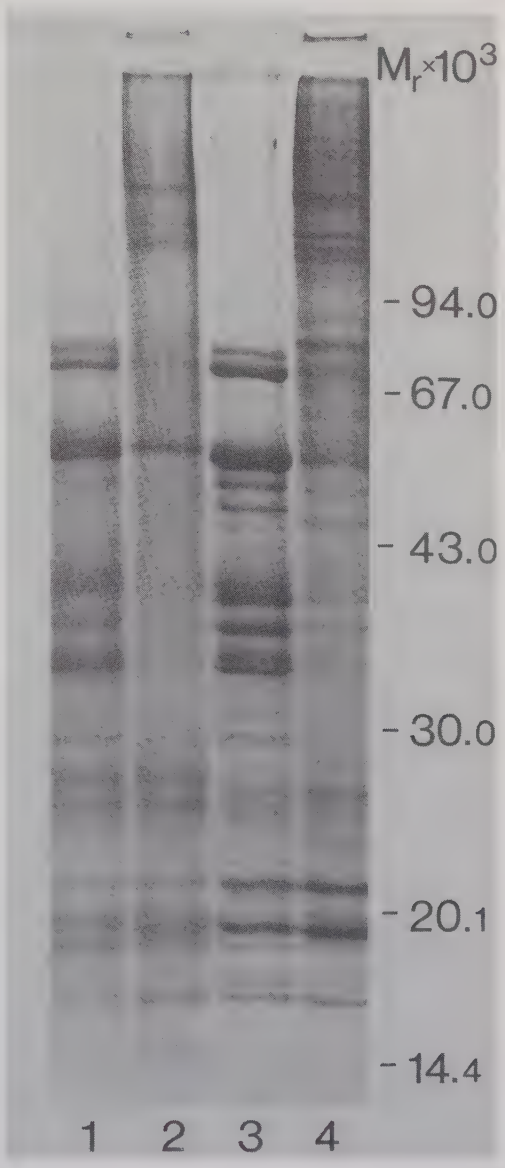


FIG. 1. SDS-PAGE with the spacer gel (5%) left on top of the gradient gel. The dotted line indicates the interface between the gradient gel and the spacer. The proteins were stained with Coomassie R 250 after completed electrophoresis. 1) Pure seminal vesicle secretion (2 μl) reduced with 10 mmol/l DTT and carboxymethylated before electrophoresis; 2) as in (1), non-reduced sample; 3) seminal clot, washed free of soluble components, reduced with 10 mmol/l DTT and carboxymethylated before electrophoresis; 4) as in (3), non-reduced sample.

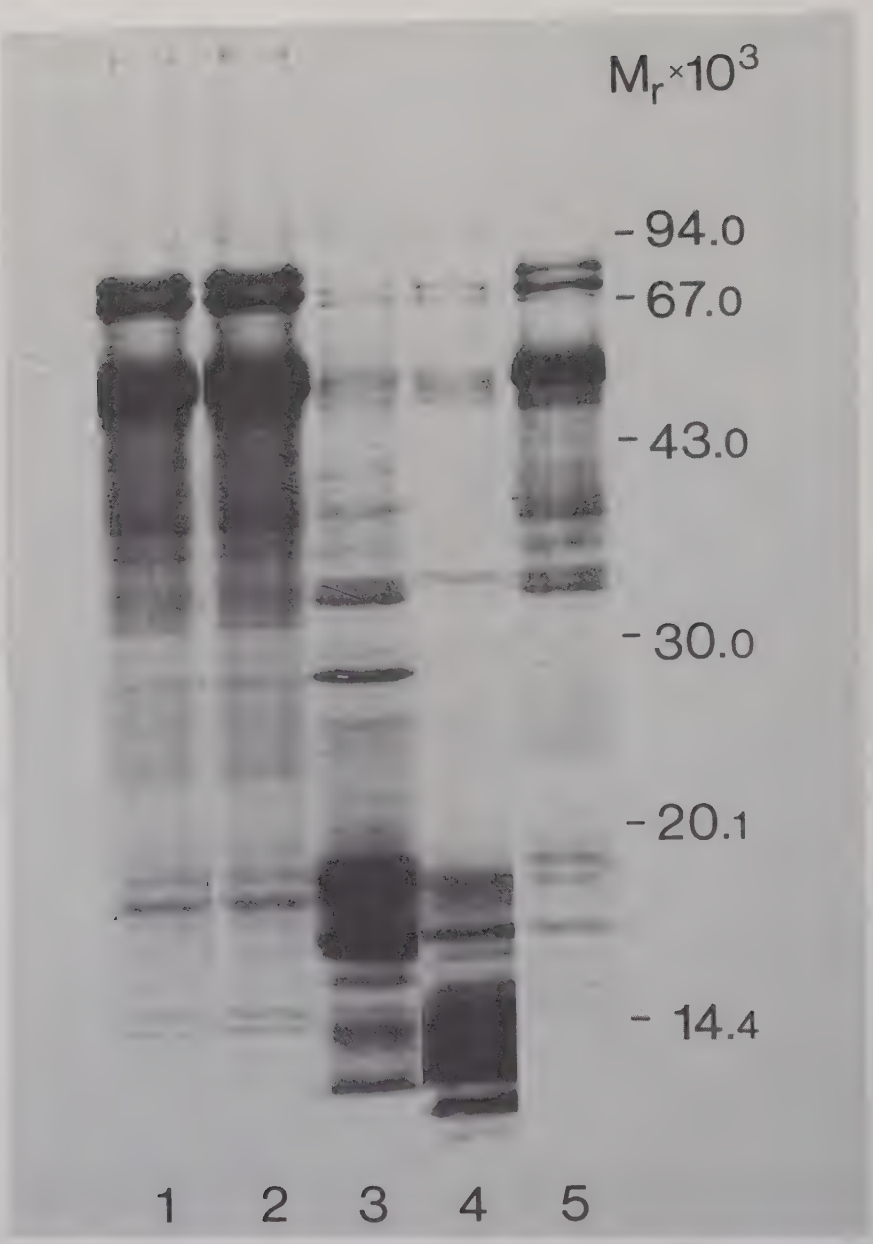


FIG. 2. SDS-PAGE with all samples reduced (10 mmol/l DTT) and carboxymethylated before electrophoresis. The proteins were stained with Coomassie R 250 after completed electrophoresis. 1) Pure seminal vesicle secretion (2 µl) stored at −70 °C after collection at operation; 2) pure seminal vesicle secretion (2 µl) incubated in vitro at 37 °C for 20 h; 3) prostatic fluid mixed with the seminal vesicle secretion (at a volume ratio of 1:4) and incubated in vitro at 37 °C for 5 min; 4) as in (3) but the sample was incubated in vitro at 37 °C for 90 min; 5) prostatic fluid that was heated to 60 °C for 10 min before mixing with the seminal vesicle secretion (at a volume ratio of 1:4) and incubated in vitro at 37 °C for 90 min.

showed that, when the samples were unreduced, a part of the protein never penetrated into the spacer gel (5% acrylamide). Using immunoblotting technique, the top surface of the spacer gel and the smeared proteins in the gradient gel were found to contain the HMW-SV-protein (not shown). Both in seminal vesicle secretion and in clotted semen, the HMW-SV-protein gave discrete bands in the gradient gel if the samples were reduced (10 mmol/l DTT) before electrophoresis. No HMW-SV-protein was retained in the spacer gel after reduction.

*4 Cleavage of HMW-SV-protein by prostatic secretory components*

In seminal vesicle secretion collected at operation, the HMW-SV-protein remained essentially uncleaved during in vitro storage for 20 h at 37 °C, as judged by SDS-PAGE (Fig. 2). The addition of prostatic fluid to the seminal vesicle secretion (at a volume ratio of 1:4) before incubation at 37 °C, resulted in complete cleavage of the HMW-SV-protein after 5 min. The HMW-SV-protein remained essentially uncleaved after 90 min at 37 °C, if the prostatic

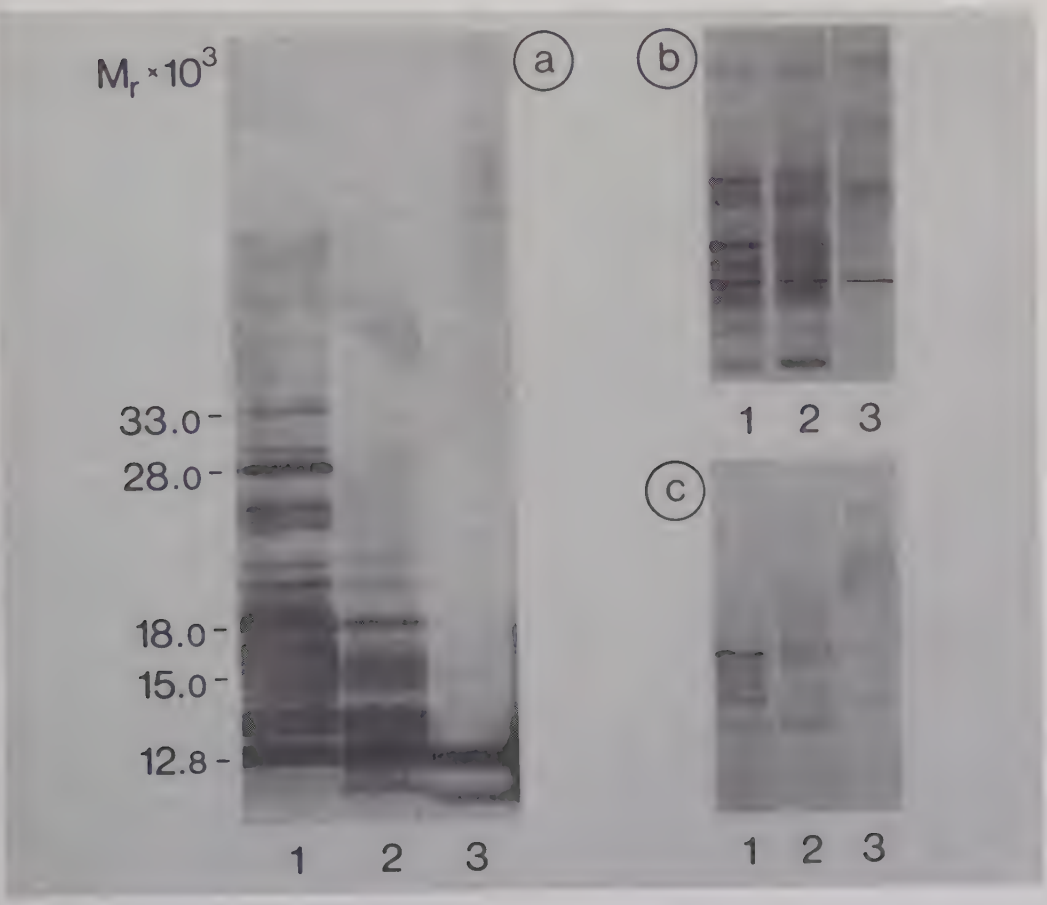


FIG. 3. (a) Immunoblotting after SDS-PAGE with all samples reduced (10 mmol/l DTT) and carboxymethylated before electrophoresis. Rabbit antiserum against the predominant basic seminal plasma protein was used as first antibody; (b) agarose electrophoresis at pH 8.6. The proteins were fixed and stained with Coomassie R 250 after completed electrophoresis. Anode at top; (c) immunoblotting after agarose electrophoresis at pH 8.6 using the same antibody as in (a). Anode at top. 1) Fluid phase of semen after in vitro incubation at 37 °C for 5 min after ejaculation; 2) as in (1) after in vitro incubation at 37 °C for 90 min after ejaculation; 3) as in (1) after in vitro incubation at 37 °C for 24 h after ejaculation.

fluid had been heated to 60 °C for 10 min before being mixed with the seminal vesicle secretion (Fig. 2). Ejaculated semen that had been frozen directly after ejaculation, but without added protease inhibitors, was incubated at 37 °C. At regular intervals seminal fluid was drawn from the incubated sample and DFP (10 mmol/l) was added immediately to arrest the proteolysis. The samples were analysed using SDS-PAGE and agarose gel electrophoresis (Fig. 3). Immunoblotting after SDS-PAGE showed the seminal fluid to contain four major HMW-SV-related proteins during the first 10 min after ejaculation. These proteins had apparent masses of 33 kDa, 28 kDa, 18 kDa, and 15 kDa respectively, and migrated towards the cathode on agarose gels during electrophoresis at pH 8.6. All four major HMW-SV-related proteins and the minor ones were labile during incubation of semen at 37 °C. The bulk of HMW-SV-related proteins, recognized after complete liquefaction of semen, had an apparent mass below 20 kDa. The most basic protein, with an apparent mass of 12.8 kDa on SDS-PAGE, became the predominant HMW-SV-related protein in semen after an incubation time of 45 to 90 min. Anodal protein, moving faster than albumin on agarose gel electrophoresis, appeared synchronously but was not recognized at immunoblotting. When semen was examined after 24 h, no HMW-SV-related proteins were detected at the cathode end of agarose gels after electrophoresis; and, according to SDS-PAGE, all HMW-SV-related proteins were smaller in size than the predominant basic protein.

*5. Staining for transglutaminase activity*

No thrombin-dependent or independent transglutaminase activity was detected either in seminal plasma, seminal vesicle secretion, or in fluid in which prostatic secretory components were predominant.

## DISCUSSION

Polymerization of basic proteins secreted by the seminal vesicles results in the clotting of ejaculated seminal fluid in rodents. These basic proteins are cross-linked via intermolecular γ-glutamyl-ε-lysine bonds. This reaction is catalysed by transglutaminase secreted by the ventral lobe of the prostate gland, the coagulating gland (reviewed by Williams-Ashman [16]). The resulting clot has a very rigid structure which, unlike the human seminal clot, does not readily dissolve. In the rat, the basic vesicular protein responsible for clot formation has been shown to form disulphide-linked, high molecular weight complexes [16]. The presented results provide evidence for the presence of disulphide-linked, high molecular weight complexes of HMW-SV-proteins in both seminal vesicle secretion and in seminal clots. This is in agreement with the results reported by Chaistitvanich & Boonsaeng [3] but cleavage of these disulphide linkages does not dissolve the clot. The human clot is, however, readily dissolved by denaturation. This indicates that non-covalent complexes of HMW-SV-proteins are essential to clot structure. Tauber et al. reported in 1976 [17] that no factor XIII was to be found in human seminal plasma using immunological methods. This is consistent with the non-covalent nature of complexes between HMW-SV-proteins, and with our finding that no transglutaminase activity was detected in any of the analysed seminal fluid specimens despite the use of a highly sensitive method.

The HMW-SV-protein was stable in pure seminal vesicle secretion but was rapidly cleaved by prostatic secretory components. This cleavage of HMW-SV-protein causes the fast shift in the agarose and SDS-polyacrylamide gel electrophoretic protein patterns, if semen is stored in vitro without protease inhibitors. Proteins with prealbumin-mobility appeared during this proteolysis but were not recognized by the antiserum raised against the predominant basic protein. However, it may be concluded from the amino acid analyses and the agarose electrophoretic patterns, that acidic peptides are released during the proteolysis of HMW-SV-protein.

We have previously reported that the addition to semen of thiol reagents, serine protease inhibitors, or o-phenanthroline, may inhibit this proteolysis [9]. A membrane-bound prostatic peptidase that hydrolyses succinyl (alanine)$_3$-p-nitroanilide [18] participates in the proteolysis of HMW-SV-protein [2]. The involvement of other proteases in the cleavage of HMW-SV-protein is indicated by the fact

that the membrane-bound peptidase is not inhibited either by DFP or by iodoacetamide [18]. A major secretory component from the human prostate, termed prostate specific antigen, was recently shown to have proteolytic activity [19]. This protein is in its $NH_2$-terminal part very similar to the $NH_2$-terminal of the predominant canine prostatic protease [19, 20]. The canine enzyme has been shown to be a serine protease [21, 22]. This might suggest the involvement of the human prostate specific antigen in the cleavage of the major seminal vesicle protein (HMW-SV-protein).

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# A KALLIKREIN-LIKE SERINE PROTEASE IN PROSTATIC FLUID CLEAVES THE PREDOMINANT SEMINAL VESICLE PROTEIN

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ABSTRACT

A 33 kD glycoprotein, known as the "prostate specific antigen". was purified to homogeneity from human seminal plasma. The prostatic protein was identified as a serine protease, and its $NH_2$-terminal sequence strongly suggests that it belongs to the family of glandular kallikreins. The structural protein of human seminal coagulum, the predominant protein in seminal vesicle secretion, was rapidly cleaved by the prostatic enzyme, which suggests that this seminal vesicle protein may serve as the physiological substrate for the protease. The prostatic enzyme hydrolyzed arginine- and lysine-containing substrates with distinct preference for the former. All synthetic substrates tested were poor substrates for the enzyme. Synthetic factor $XI_a$ substrate (pyro-glutamyl-prolyl-arginine-p-nitroanilide), and the synthetic kallikrein substrate (H-D-prolyl-phenylalanyl-arginine-p-nitroanilide) were hydrolyzed with maximum specific activities at 23° C of 79 nmol/min/mg and 34 nmol/min/mg and $K_m$ values of 1.0 and 0.45 mM, respectively. Synthetic substrates for plasmin, chymotrypsin, and elastase were either not hydrolyzed by the enzyme at all, or only very slowly.

*Abbreviations used:* HMW-SV-protein, high molecular weight seminal vesicle protein; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; DFP, di-isopropylfluorophosphate; NPGB, 4-nitrophenyl-guanido-benzoate; pNA, 4-nitroanilide; Suc, succinyl; DTT, dithiothreitol; Con A, concanavalin A.

A seminal coagulum forms immediately after the ejaculation of human semen. The predominant protein in seminal vesicle secretion constitutes the structural protein of the clot (1). This protein, known as the HMW-SV-protein, forms disulfide-linked high molecular weight complexes but gives major protein bands of some 52, 71, and 76 kD on SDS-PAGE in the presence of reducing agents (2, 3). The HMW-SV-protein is cleaved by proteases in the prostatic fluid to several low molecular weight proteins in parallel with the liquefaction of the clot (1, 3). A predominant cleavage product of the HMW-SV-protein is a 5.8 kD basic protein that in its $NH_2$-terminal portion is similar to the histidine-rich region of bovine HMW-kininogen (4, 5, 6). In the rat, the $NH_2$-terminal portion of this basic protein is also reported to inhibit the secretion of pituitary follicle stimulating hormone (7).

A 30 kD arginyl-cleaving serine protease is predominant in canine prostatic fluid (8, 9). Sequence homologies with glandular kallikreins strongly suggest that the canine protease is a member of the kallikrein family (10). A secretory protein from the human prostate, known as "prostate specific antigen", has recently been shown to be a proteolytic enzyme (11). This 33 kD glycoprotein is present in human prostatic fluid in high concentration (12). Comparison of the reported $NH_2$-terminal sequences of the human prostatic antigen (11) and of the canine protease (10) suggests that these proteins are species analogues. If this

assumption is correct, the human prostatic antigen should be a kallikrein-like protease that might cleave a kininogen-related substrate like the HMW-SV-protein. The present investigation presents results that support this hypothesis.

## METHODS

*Reagents:* Unlabeled DFP, NPGB-HCl, and $N^{\alpha}$-benzoyl-L-tyrosine ethyl ester were obtained from Sigma Chemical Co. (St. Louis, Mo., USA); ($^{3}$H)-DFP from Amersham Corp. (Amersham, UK); ENHANCE from New England Nuclear (Dreieich, FRG); pyro-glutamyl-prolyl-arginine-pNA (S 2366), H-D-prolyl-phenylalanyl-arginine-pNA (S 2302), H-D-valyl-leucyl-arginine-pNA (S 2266), H-D-phenylalanyl-pipecolyl-arginine-pNA (S 2238), and H-D-valyl-leucyl-lysine-pNA (S 2251) from Kabi (Stockholm, Sweden); $N^{\alpha}$-benzoyl-arginine-pNA from British Drug Houses (Poole, UK); and Suc-(alanine)$_3$-pNA from the Protein Research Foundation (Minoh, Osaka, Japan); CM-Sepharose, DEAE-Sepharose and Con A-Sepharose from Pharmacia (Uppsala, Sweden); and Ultrogel Aca 54 from LKB (Bromma, Sweden).

*Material:* Human semen, and pure prostatic fluid were obtained as previously described (13). Pure seminal vesicle secretion was aspirated from the vesicular ducts during an operation of a male patient for carcinoma in the urinary bladder (3). Coagulated semen was collected in vessels containing 0.5 ml of 0.5 M *O*-phenanthroline that were placed in ice-cold $H_2O$ directly after ejaculation and then frozen at $-70^{\circ}$ until

required (1). The coagulated semen was washed free of soluble components and the washed clots were dissolved, reduced and carboxymethylated as previously described (3). Carboxymethylated clots were extensively dialyzed against 50 mM Tris-HCl with 0.5 M NaCl, pH 8.0.

*Electrophoretic techniques:* Agarose gel electrophoresis was performed according to standard procedures (14). SDS-PAGE in a 9-17% polyacrylamide gel gradient was performed according to Blobel and Dobberstein (15). Gels with radioactive proteins were dried after protein fixation and treatment with ENHANCE according to the manufacturer's instructions, and then exposed for 30 days to X-ray films at -70° C before development. Throughout the purification procedures the individual fractions were analyzed with electroimmunoassay using a polyclonal rabbit antiserum raised against the purified prostatic 33 kD glycoprotein. The electroimmunoassay was run at 60 V for 16 h in gels that contained 1% of antiserum.

*Protein purification:* The 33 kD prostatic glycoprotein was purified from 50 ml of pooled seminal plasma. The seminal plasma pool was diluted to 150 ml and made up to 10 mM with benzamidine. Concentrations on Diaflo$^{R}$ membranes (cut-off range = 5 kD) and all chromatographic steps were carried out at 4° C. All buffers used were made up to 10 mM with benzamidine. The seminal plasma pool was applied to a CM-Sepharose column (40 x 2.5 cm) equilibrated with 50 mM Tris-HCl, pH 7.5. The prostatic glycoprotein passed through the column with the equilibrating buffer. Fractions containing the protein were

pooled and concentrated to about 100 ml. This pool was applied to a DEAE-Sepharose column (30 x 2.5 cm) equilibrated with 30 mM sodium phosphate, pH 6.4. The prostatic glycoprotein passed through the column with the equilibrating buffer and the fractions were pooled and concentrated. The pool was subsequently applied to a Con A-Sepharose column (16 x 2.5 cm) equilibrated with 50 mM Tris also containing 0.5 M NaCl, pH 7.5. The prostatic glycoprotein was eluted from the column with 0.2 M α-methyl-mannoside added to the equilibrating buffer and the fractions were pooled and concentrated (Fig. 1 a). The protein pool was applied to an Ultrogel Aca 54 column (88 x 1.6 cm) equilibrated with 50 mM Tris and 0.5 M NaCl, pH 8.0 (Fig. 1 b). Fractions reacting with the antibody were pooled and the resulting material found to contain a single band of about 33 kD as judged by SDS-PAGE of reduced samples (Fig. 2 a). The recovery after each purification step is given in Table 1.

*Enzyme assays:* Hydrolysis of the tripeptide pNA-substrates and $N^{\alpha}$-benzoyl-arginine-pNA was measured with a Shimadzu UV 260 recording spectrophotometer at 405 nm. Hydrolysis of $N^{\alpha}$-benzoyl-L-tyrosine ethyl ester was measured at 253 nm. All incubations were performed at $23^{o} \pm 2^{o}$ C in 50 mM Tris, pH 7.8. The reaction was initiated by the addition of the purified protein to 1 ml of substrate solution, while the absorbance was monitored for at least 20 min against a blank containing the substrate solution.

*Labeling with ($^3H$)-DFP:* Samples of purified protein (each containing about 4 µg), pure prostatic fluid (4 µl), and seminal plasma (4 µl) were incubated with 3 µl of ($^3H$)-DFP (5 mCi/ml) in 15 µl of 50 mM Tris-HCl, pH 7.8, for 10 to 30 min at room temperature. The samples were subsequently analyzed using SDS-PAGE after reduction and carboxymethylation.

*Titration of enzymatic activity:* Active site titration with NPGB was performed according to Chase and Shaw (17). The molar concentration of the purified protein was calculated from amino acid analysis assuming a mass of 33 kD and a carbohydrate content of 10% (12).

*Amino acid analysis and sequence determination:* The purified protein was reduced for 1 h with 25 mM DTT in 0.5 M Tris-HCl with 6 M guanidine-HCl, pH 8.0, and then carboxymethylated for 2 h with 50 mM iodoacetic acid in 0.5 M Tris, pH 8.0. Carboxymethylated protein was hydrolyzed in 6 M HCl in evacuated sealed tubes at 110° C for 24 h: The hydrolysates were analyzed in a Beckman 6300 amino acid analyzer. The $NH_2$-terminal sequence of about 40 µg of carboxymethylated protein was analyzed using an Applied Biosystems 470A gas phase sequencer with a standard program (18). All PTH-amino acids were identified with HPLC on a Novapak $C_{18}$ column (Waters). The column was equilibrated in 29 mM sodium acetate, pH 5.05, with 17% acetonitrile and eluted with a linear gradient against 60% isopropanol.

RESULTS

*Identification of the purified protein*

Analysis of the fifteen $NH_2$-terminal amino acid residues of the purified protein gave the following sequence: Ile-Val-Gly-Gly-Trp-Glu-Cys-Glu-Lys-His-Ser-Gln-Pro-Trp-Gln. The yield of the first amino acid (0.9 nmol of Ile) corresponded to about 75% of the calculated theoretic yield.

*Enzymatic activity of the purified protein*

The purified protein did not hydrolyze $N^{\alpha}$-benzoyl-tyrosine ethyl ester or Suc(alanine)$_3$pNA. Kinetic data for various synthetic substrates are given in Table 2. DFP added to the purified protein in 200 fold molar excess abolished its hydrolysis of H-D-prolyl-phenylalanyl-arginine-pNA and pyro-glutamyl-prolyl-arginine-pNA. Active site titration of the purified protein with NPGB demonstrated that 66% of the protein had enzymatic activity.

*Incorporation of ($^3$H)-DFP in the purified protein*

Fluorographic enhancement after SDS-PAGE of the purified protein showed that it incorporated ($^3$H)-DFP in a time dependent manner (Fig. 2 b). The incorporation of ($^3$H)-DFP was blocked if the purified protein had been treated with unlabeled DFP (10 mM) or if it had been carboxymethylated before it was incubated with the radioactive compound. One single band, with the same mass as the purified protein, incorporated ($^3$H)-DFP both in pure prostatic secretion and in seminal plasma as judged by SDS-PAGE (Fig. 2 b).

*Cleavage of HMW-SV-protein by the purified protein*

Native HMW-SV-protein (in seminal vesicle secretion), and carboxymethylated HMW-SV-protein (from seminal clots) were analyzed by SDS-PAGE (Fig. 3). The intact HMW-SV-protein gave three major bands of 52, 71, and 76 kD as marked with arrows in the figure. The series of minor bands with molecular masses below 50 kD (lanes 1 and 6 in Fig. 3) are intermediate cleavage products of the HMW-SV-protein as previously demonstrated in seminal clots by immunoblotting after SDS-PAGE (1, 3). The seminal vesicle secretion and seminal clots were incubated at 37° C with the purified 33 kD prostatic protein (3 µg). The purified protein rapidly cleaved native or carboxymethylated HMW-SV-protein as shown by the fast disappearance of the 52 and 76 kD bands. The 71 kD band of the intact protein was somewhat more resistant to cleavage (lanes 2-4 and 7 in Fig. 3). Below the weakly stained 71 kD band of HMW-SV-protein in lane 7 a faint 67 kD band is seen which is albumin. Proteins with molecular masses below 20 kD appeared in parallel with the disappearance of intact HMW-SV-protein. On agarose gel electrophoresis the cleavage of HMW-SV-protein by the purified prostatic protein gave a series of basic proteins that were indistinguishable from the basic proteins of liquefied semen (Fig. 4). The basic low molecular mass degradation products are HMW-SV-related proteins as previously shown by immunoblotting (1, 3). In addition, there also appeared acidic cleavage products when seminal clots (Fig. 4) or seminal vesicle secretion (not shown) were incubated with the purified prostatic protein. The 5.8 kD predominant basic protein in seminal plasma was one of the end products formed after 60 min (Figs. 3 and 4). However, purified 5.8 kD basic protein was not cleaved further by the purified protein (not shown).

No cleavage of intact HMW-SV-protein occurred if the purified protein (3 µg) had been pretreated with DFP (in 200 fold molar excess) before the incubation with seminal clots was initiated (lane 5 in Fig. 3).

## DISCUSSION

The purified protein had a molecular mass of about 33 kD and contained carbohydrate as judged by the high affinity for con A. Its $NH_2$-terminal sequence was identical to that reported for "prostate specific antigen" (11). In conjunction with the prostatic origin of our purified protein, these results provide evidence of its identity with the prostate specific antigen. Ban et al. (11) reported that prostate specific antigen possessed proteolytic activity, but that it not was a serine protease although the $NH_2$-terminal sequence was very similar to serine proteases, in particular rat submaxillary tonin (19). Our results clearly demonstrate that the purified protein was a serine protease. DFP destroyed the functional activity of the purified protein. It incorporated ($^3H$)-DFP in a specific and time dependent manner. The result from titration of enzymatic activity excludes the possibility that the demonstrated ($^3H$)-DFP incorporation may have been due to a minor contaminant present in the purified material. Furthermore, the sequence (-Val-Leu-Thr-Ala-Ala-His-Cys-) reported for prostate specific antigen by Ban et al. (11) suggests that His 41 is a member of the catalytic triad of serine proteases (20). The purified protein had no chymotryptic (tyrosine-cleaving) or elastolytic (alanine-cleaving) activity. It possessed a

substrate specificity restricted to arginine- and lysine-containing substrates, but with a distinct preference for the former. Synthetic plasma kallikrein-substrate (Pro-Phe-Arg-pNA) and factor $XI_a$-substrate (Glu-Pro-Arg-pNA) were the most efficient but all synthetic substrates tested were poor substrates for the purified protein. However, the prostatic enzyme very rapidly cleaved intact HMW-SV-protein, as judged by the fast disappearance of the 52, 71, and 76 kD bands on SDS-PAGE. The presented results do not permit description of one separate band being transformed to another due to the complexity of the HMW-SV-protein cleavage. The enzyme to substrate concentration ratio used for the electrophoretic mapping of the HMW-SV-protein cleavage by the purified enzyme was comparable to their physiological ratio in ejaculated semen. The alterations of the protein pattern resulting from cleavage of HMW-SV-protein by the prostatic enzyme were very similar to the normal proteolysis of HMW-SV-protein in semen (3). It may, therefore, be concluded that the HMW-SV-protein serves as a (or perhaps the) physiological substrate for the purified prostatic protein when prostatic fluid is mixed with the seminal vesicle secretion.

The reported $NH_2$-terminal sequence for prostate specific antigen (11), consisting of 68 amino acids, is to 37% identical to porcine trypsin (21), but the identity to both porcine pancreatic kallikrein (22) and to rat tonin (19) is 54%. These sequence homologies strongly suggest that our 33 kD prostatic serine protease (prostate specific antigen), is more similar to the family of glandular kallikreins than to trypsin. The predominant canine prostatic enzyme is a serine protease that, according to partial sequence analysis, also

has been suggested as being a member of the glandular kallikrein family (10). This canine enzyme has similar specificities for synthetic substrates as compared with our human protease (8). The canine protease is reasonably the species analogue to our purified protein as their masses and $NH_2$-terminal sequences are very similar (10, 11).

The $NH_2$-terminal portion of the predominant 5.8 kD cleavage product of the HMW-SV-protein has sequence homologies with two portions of the histidine-rich region of bovine HMW kininogen (4). The data presented here indicate that a system consisting of a kallikrein-like enzyme and a kininogen-like substrate is activated at the ejaculation of human semen. It is of pertinent interest to study the sperm interactions of the liberated cleavage products of the HMW-SV-protein.

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LEGENDS TO FIGURES

Fig. 1 a. Affinity chromatography on Con A-Sepharose at a flow rate of 25 ml/h of pooled fractions from DEAE-Sepharose containing the prostatic 33 kD glycoprotein. Elution with 0.2 M α-methyl-D-mannoside was started at ↓ . The hatched peak contained the 33 kD glycoprotein.

Fig. 1 b. Gel filtration on Ultrogel AcA 54 at a flow rate of 3 ml/h of the pooled fractions from above. The fractions that were pooled, concentrated, and analyzed by SDS-PAGE in Fig. 2 a have been indicated by hatching.

Fig. 2 a. SDS-PAGE in a 9-17% polyacrylamide gel gradient. All samples were reduced (10 mM DTT) and carboxymethylated before electrophoresis. The proteins were stained with Coomassie R 250 after completed electrophoresis.

1. Purified prostatic protein (about 15 µg)
2. Prostatic fluid (4 µl)

Fig. 2 b. Fluorographic enhancement after SDS-PAGE.

1. Purified protein (5 µg) incubated with ($^{3}$H)-DFP for 10 min.
2. Purified protein (5 µg) incubated with ($^{3}$H)-DFP for 30 min.

3. Purified protein (5 µg) treated with unlabelled DFP (10 mM) before incubation with ($^3$H)-DFP for 30 min.
4. Purified protein (5 µg) reduced and carboxymethylated before incubation with ($^3$H)-DFP for 30 min.
5. Prostatic fluid (4 µl) incubated with ($^3$H)-DFP for 10 min.
6. Prostatic fluid (4 µl) incubated with ($^3$H)-DFP for 30 min.
7. Seminal plasma (4 µl) incubated with ($^3$H)-DFP for 30 min.

Fig. 3. SDS-PAGE in a 9-17% polyacrylamide gel gradient. All samples were reduced (10 mM DTT) and carboxymethylated before electrophoresis. The proteins were stained with Coomassie R 250 after completed electrophoresis.

1. Dissolved, reduced and carboxymethylated seminal clot (10 µl).
2. Purified prostatic protein (3 µg) incubated with the carboxymethylated clot (10 µl) at 37° C, for 2 min.
3. As in (3) but the sample was incubated for 15 min.
4. As in (3) but the sample was incubated for 60 min.
5. Purified prostatic protein (3 µg) treated with DFP (10 mM) before it was mixed with the carboxymethylated clot (10 µl) and incubated at 37° C for 30 min.

6. Pure seminal vesicle secretion (2 µl).
7. Purified prostatic protein (3 µg) incubated with the seminal vesicle secretion (2 µl) at 37° C, for 5 min.
8. Purified prostatic protein (5 µg).
9. Purified 5.8 kD predominant basic protein.

Fig. 4. Agarose gel electrophoresis at pH 8.6. The proteins were fixed and stained with Coomassie R 250 after completed electrophoresis. Anode at top.

1. Liquefied semen (4 µl).
2. Purified prostatic protein (3 µg).
3. Dissolved, reduced and carboxymethylated seminal clot (10 µl).
4. Purified prostatic protein (3 µg) incubated with the carboxymethylated clot (10 µl) at 37° C, for 60 min.
5. Purified 5.8 kD predominant basic protein.

Table 1

Purification of prostatic 33 kD glycoprotein.

| Steps | Total volume ml | Total protein mg | Total amount of 33 kD glycoprotein mg | Recovery % |
|---|---|---|---|---|
| Seminal plasma | 50 | 2300 | 63 | 100 |
| CM-Sepharose | 100 | 600 | 52 | 82 |
| DEAE-Sepharose | 7.0 | 130 | 26 | 41 |
| Con A-Sepharose | 4.0 | 32 | 18 | 28 |
| Ultrogel AcA 54 | 4.2 | 13 | 12 | 19 |

The amount of prostatic 33 kD glycoprotein was determined by electro-immunoassay. The assay was calibrated by the use of amino acid analysis of the pooled fraction from AcA 54. Total protein was determined according to Lowry (16) with the use of albumin as standard.

Table 2

Hydrolysis of synthetic substrates by the purified protein. All assays were performed at $23^{o} \pm 2^{o}$ C and pH 7.8 as described in methods. Michaelis-Menten constants were obtained from Lineweaver-Burk plots of rates of hydrolysis. Hydrolysis of H-D-valyl-leucyl-lysine-pNA and $N^{\alpha}$-benzoyl-arginine-pNA were measured at a substrate concentration of 1 mM.

| Substrate | Vmax nmol/min/mg | Km mM | Kcat $s^{-1}$ | Kcat/Km $s^{-1}M^{-1}$ |
|---|---|---|---|---|
| Pyro-glutamyl-prolyl-arginine-pNA | 79 | 1.0 | 0.064 | 64 |
| H-D-prolyl-phenylalanyl-arginine-pNA | 34 | 0.45 | 0.028 | 62 |
| H-D-phenylalanyl-pipecolyl-arginine-pNA | 36 | 0.59 | 0.029 | 49 |
| H-D-valyl-leucyl-arginine-pNA | 34 | 4.0 | 0.028 | 7 |
| H-D-valyl-leucyl-lysine-pNA | 2.9 | - | - | - |
| $N^{\alpha}$-benzoyl-arginine-pNA | 7.5 | - | - | - |

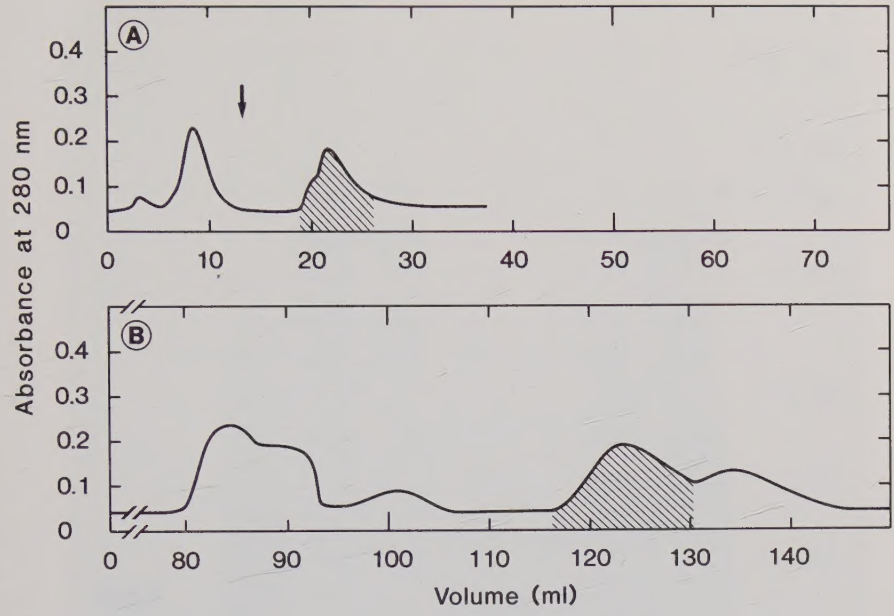
A
B
Absorbance at 280 nm
0.4
0.3
0.2
0.1
0
0 10 20 30 40 50 60 70
0 80 90 100 110 120 130 140
Volume (ml)

Fig. 1

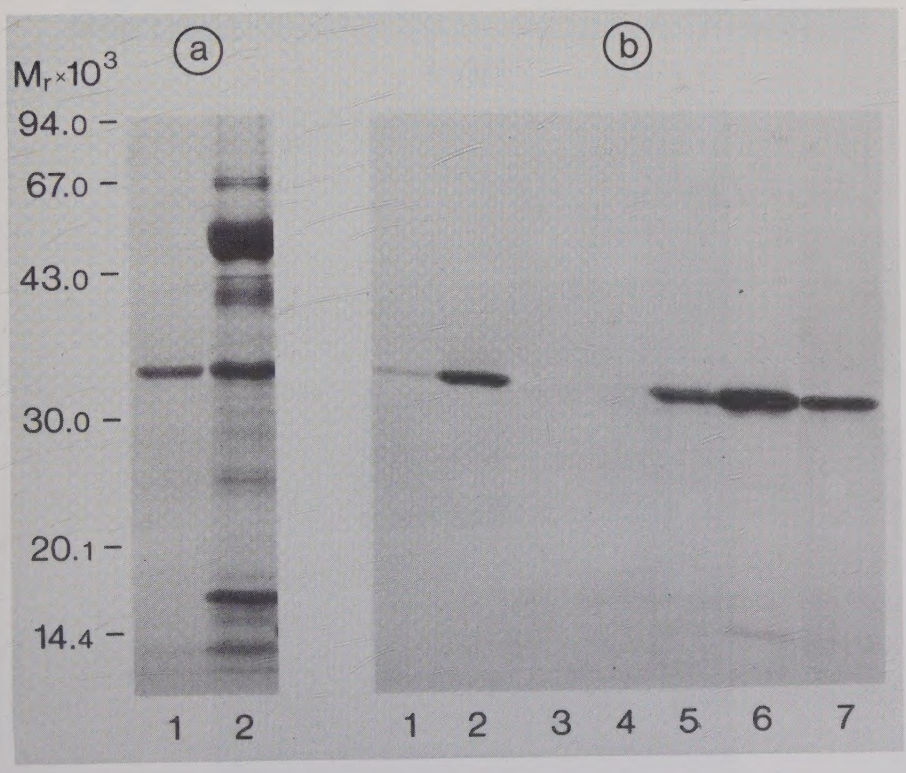
a
b
Mr×10^3
94.0
67.0
43.0
30.0
20.1
14.4
1 2
1 2 3 4 5 6 7

Fig. 2

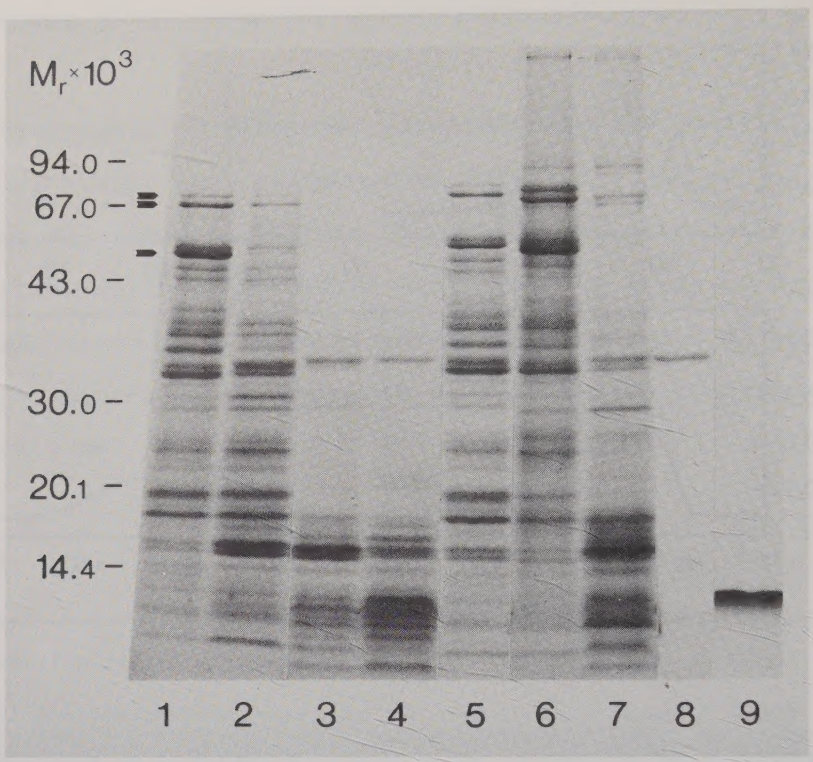


Fig. 3

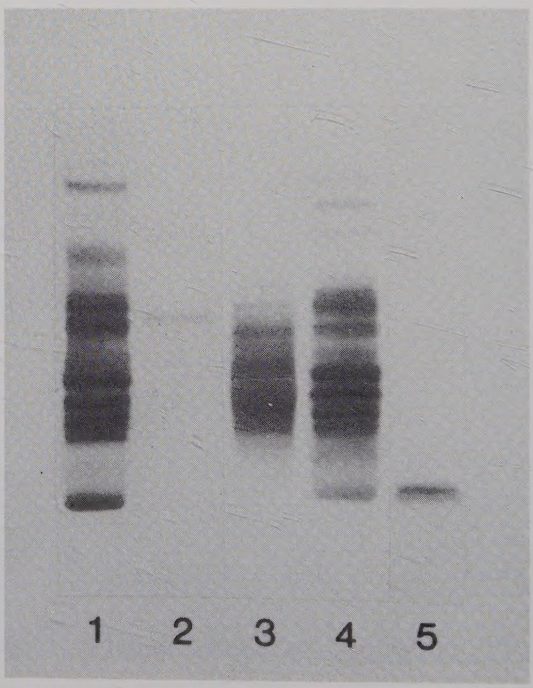


Fig. 4

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